THE ROLE OF LIPID SIGNALING AND METABOLISM IN MORPHOGENESIS AND PATHOGENESIS OF THE FUNGAL PATHOGEN *USTILAGO MAYDIS*

by

JANA KLOSE

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ABSTRACT

The phytopathogenic fungus *Ustilago maydis* is obligately dependent on infection of maize to complete the sexual phase of its life cycle. Mating interactions between budding cells establish an infectious filamentous cell type that invades the host, induces tumors, and forms teliospores. The yeast-to-filamentous morphological transition is regulated by cAMP and MAPK signaling pathways known to control the pathogenic development in the host. The signals influencing this transition during infection have not yet been identified. In this study, we demonstrated that lipids triggered the dimorphic switch to promote a filamentous phenotype resembling the infectious filaments found *in planta*, which was dependent on cAMP and Ras/MAPK signaling. In addition, low levels of lipids (4nM) induced the response suggesting that they are acting as ligands to trigger the morphological change. Overall, lipids may represent one of the signals that promotes and maintains filamentation of the fungus in the host.

To explore potential metabolic and signaling roles of lipids in morphogenesis and pathogenesis, we deleted genes encoding enzymes in the β-oxidation of fatty acids (*mfe2*, peroxisomal multifunctional enzyme; *had1*, mitochondrial 3-hydroxyacyl-CoA dehydrogenase) and a phospholipase A2 (*lip2*). Loss of *mfe2* blocked extensive proliferation of fungal filaments *in planta*, delayed sporulation and reduced virulence. Loss of *had1* resulted in attenuation of disease symptoms and impaired teliospore germination. These findings suggest that mitochondrial β-oxidation may be crucial during teliospore germination and initial stages of *in planta* fungal development, and that peroxisomal β-oxidation may be required during later stages of *in planta* development. In addition, Mfe2 and Had1 were specifically required for the filamentation induced by linoleic and myristic acid, respectively. Overall, lipids represent an important carbon source during biotrophic growth, and lipid utilization by *U. maydis* may influence additional aspects of infection (i.e., signal perception or host defense). Loss of *lip2* resulted in more severe symptom development and more rapid teliospore maturation during infection. The Lip2 function might be important during fungus-host interactions to limit premature development of disease symptoms prior to sporulation. In summary, this work contributes to the emerging idea that lipid metabolism and signaling are important for biotrophic interactions between plants and fungal pathogens.
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LIST OF ABBREVIATIONS

aa       amino acid
ABC      ATP binding cassette
AC       adenylyl cyclase
ATP      adenosine triphosphate
BLAST    Basic Local Alignment Search Tool
C        Celsius
cAMP     cyclic adenosine mono-phosphate
CM       complete medium
CoA      coenzyme A
DAG      diacylglycerol
DIC      differential interference contrast
HAD      3-hydroxyacyl-CoA dehydrogenase
hr       hour
hyg      hygromycin
JA       jasmonic acid
kb       kilobase pair
LCFA     long-chain fatty acids
LDS      linoleate diol synthase
MAPK     mitogen activated kinase
MCFA     medium-chain fatty acids
uL       microliter
mL       milliliter
nat      nourseothricin
ORF      open reading frame
PAF_AH   platelet-activated factor_acylhydrolase
PC       phosphatidylcholine
PCR      polymerase chain reaction
PDA      potato dextrose agar
PDB      potato dextrose broth
PI       phosphatidylinositol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLA1</td>
<td>phospholipase A1</td>
</tr>
<tr>
<td>PLA2</td>
<td>phospholipase A2</td>
</tr>
<tr>
<td>PLB</td>
<td>phospholipase B</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>phospholipase D</td>
</tr>
<tr>
<td>PRE</td>
<td>pheromone response element</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SA</td>
<td>salicylic acid</td>
</tr>
<tr>
<td>SAGE</td>
<td>Serial Analysis of Gene Expression</td>
</tr>
<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
</tr>
<tr>
<td>SCFA</td>
<td>short-chain fatty acids</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloric acid</td>
</tr>
<tr>
<td>TGL</td>
<td>triglyceride lipase</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>VLCFA</td>
<td>very long-chain fatty acids</td>
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</table>
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CO-AUTHORSHIP STATEMENT

The work presented herein is the culmination of research from 2001 to 2006. Below is the list of papers that have been published/accepted/in preparation as a result of this work, and the contribution made by the candidate:

Chapter 2


The candidate is responsible for all of the work completed in this chapter. The second author has made the initial observation that plant oils trigger filamentation in *U. maydis*, on which the work described in this paper is based. The writing of the manuscript was completed by Dr. Kronstad, who acted as a supervisor for this project, and the candidate.

Chapter 3


The work detailed in this chapter was completed solely by the candidate. The writing of the manuscript was completed by Dr. Kronstad, who acted as a supervisor for this project, and the candidate.

Chapter 4

Klose, J., and Kronstad, J. W. Had1 is required for teliospore germination and may play a role in early stages of *in planta* fungal development in *U. maydis* (in preparation)

The work detailed in this chapter was completed solely by the candidate. The writing of the manuscript is being completed by Dr. Kronstad, who acted as a supervisor for this project, and the candidate.
Chapter 5

Klose, J., and Kronstad, J. W. Phospholipase A2 attenuates the disease symptom development during infection of *U. maydis* (in preparation)

The work detailed in this chapter was completed solely by the candidate. The writing of the manuscript is being completed by Dr. Kronstad, who acted as a supervisor for this project, and the candidate.
1. INTRODUCTION

1.1. Plant infection and fungal biotrophy

Biotrophic pathogens such as the maize smut fungus *Ustilago maydis* must grow on their hosts to propagate and cause disease. This interaction is typified by an intimate relationship in which host cells stay alive while metabolites are redirected to feed the pathogen. Biotrophic interfaces are formed in symbiotic and parasitic plant-fungal interactions. They result from coordinated developmental programs in both partners and represent specialized platforms for the exchange of information and nutritional metabolites. Three types of biotrophy can be distinguished: obligate, semi-obligate and hemi-obligate biotrophy. Semi-obligate biotrophic fungi such as *U. maydis* develop a biotrophic relationship with their host that lasts for nearly all the life cycle of the fungus. Often, an early phase of the life cycle takes place outside of a host and the life cycle continues only if a host plant is available to the fungus. Hemiobligate-biotrophic fungal pathogens have an initial biotrophic phase that is followed by a necrotrophic phase, in which the fungus kills the host plant cells and feeds off the dead tissue. Much of what we know about biotrophic fungi and their metabolism comes from the studies on hemi-obligate biotrophs such as *Cladosporium fulvum* (Thomma et al., 2005), *Magnaporthe grisea* (Talbot, 2003), and *Mycosphaerella graminicola* (Palmer and Skinner, 2002). Much less is known about the obligate biotrophs, such as the rust fungi or powdery mildews, which only grow in association with host plants. Biotrophic relationships are also established in symbiotic host-fungus interactions involving mycorrhizal fungi (Bago and Becard, 2002).

Successful colonization of host tissues by biotrophic pathogens depends on their ability to utilize the available nutrient sources offered by plants as well as on their ability to penetrate plants and evade defensive mechanisms. Biotrophic fungi form specialized infectious structures to achieve critical stages during pathogenesis, including attachment, host recognition, penetration, proliferation and nutrition, which are controlled by complex regulatory pathways (Kahmann and Basse, 2001; Lee et al., 2003). Some of the early observations of morphological changes that take place during fungal infection were described as early as 1899 by the botanist Anton deBary (1866). He observed that plant...
parasitic fungi alter their hyphal morphology in response to structural and physiological features of the host surface soon after germination. The early stages of infection, such as adhesion, appressorium (i.e., a round melanized penetration structure) formation and penetration (reviewed for several plant pathogenic fungi in Hardham, 2001; Tucker and Talbot, 2001) ensure reliable infection while causing minimal damage to the host cells. At this stage, pathogenic fungi undergo various morphological changes to assist in penetration of a plant cell surface. For example, in rust fungi, the tip of dikaryotic germ tube can follow topographical features of the plant cuticle to find stomatal openings where they enter host tissue (Staples and Hoch, 1997.). Similarly, chemical signals such as leaf alcohols can also contribute to the unique recognition system between a fungus and a host plant surface (Collins et al., 2001). In a phytopathogen Colletotrichum gloeosporioides, plant surface waxes act as signals that trigger germination and appressorium formation (Kolattukudy et al., 1995). In other fungi such as M. grisea, host surface and rapid mobilization of lipid and/or carbohydrate reserves influence the appressorium formation, which differentiates from the end of a fungal germ tube after spore germination on a plant surface. After the penetration of plant epidermal cells, biotrophic fungi must establish themselves within host tissue and access nutrients from the host.

Biotrophic fungi use different strategies to gain access to host nutrients. The biotrophic life style is achieved in many ways: intercellular (C. fulvum); subcuticular (Venturia inaequalis); inter- and intracellular (Claviceps purpurea, U. maydis); extracellular with haustoria within epidermal cells (powdery mildews); and intercellular with haustoria (i.e., feeding structures) within parenchyma cells (rust fungi and downy mildews). As mentioned, hemiobligate biotrophic fungi (M. grisea, Phytophtora infestans, Colletotrichum spp.) grow initially as biotrophs and switch to necrotrophic growth later in development. Very little is known about fungal nutritional requirements during their biotrophic phase. There is some evidence that phytopathogens can metabolize all the major substrates in vitro (Bailey et al., 2000; Jennings and Lysek, 1996; Lehtinen, 1993; Murphy and Walton, 1996; Noeldner et al., 1994). However, it is difficult to assess nutritional requirements during biotrophic host-fungus interactions, especially those of obligate pathogens, because they cannot be grown in culture. Because
these fungi can grow only in association with a plant, the regulation of nutrient assimilation and associated enzymes must be dependent on factors or signals produced by the plant. Recently, genomic studies have indicated that the obligate biotroph *Blumeria graminis* (powdery mildew) expresses genes required for glycogen breakdown, glycolysis, lipolysis, β-oxidation and the TCA cycle (Both et al., 2005; Thomas et al., 2002; Thomas et al., 2001). Targeting potential weakness in fungal metabolism may lead to a discovery of new antifungal strategies.

Successful biotrophic interactions between a fungus and its host also require the ability of a fungus to bypass the plant defense mechanisms. Plants are resistant to the majority of potential pathogens that they are in constant contact with, due to the inability of the pathogen to avoid recognition or to suppress plant defense. The infectious structures such as appressoria, penetration hyphae and infection hyphae are formed to invade the plant with minimal damage to the host cells. To establish compatibility with the host, controlled secretion and distinct interface layers appear to be essential (Hahn and Mendgen, 2001). Highly developed infectious structures, limited secretory activity (especially of lytic enzymes), carbohydrate-rich and protein-containing interfacial layers that separate fungal and plant plasma membranes, and long-term suppression of host defense are the hallmarks of biotrophic fungi. Properties such as the production of toxins, the elaboration of cell wall degrading enzymes, specialized structures for host penetration, specific signal transduction components and avirulence genes are also well characterized as pathogenicity factors in these systems (Idnurm and Howlett, 2001).

1.2. Morphogenesis and pathogenesis in *Ustilago maydis*

1.2.1. The biotrophic life style of *U. maydis*

*U. maydis* displays dimorphic growth alternating between a budding haploid form and a filamentous dikaryon that results from the mating of two haploid cells. This morphological transition corresponds to a change from saprophytic to pathogenic development. *U. maydis* is a semi-obligate parasite because the haploid form can be propagated on artificial media, but the fungus requires plant tissue for proliferation and sporulation after the formation of the filamentous dikaryon. Upon host infection, *U.*
*maydis* induces the production of large tumors that provide an optimal environment for massive hyphal proliferation, differentiation of sporogenic hyphae and karyogamy, and finally production of darkly pigmented diploid teliospores.

It has been hypothesized that the establishment of the biotrophic phase in the *U. maydis*/host plant interaction involves redirection of host metabolism to the site of fungal growth. The intimate relationship between *U. maydis* and its host implies that the fungus has evolved highly specialized mechanisms to recognize and adapt to its particular host. It is generally accepted that *U. maydis* is not systemically distributed but that it is localized to meristematic areas of the plant (Christensen, 1963). The regulation of developmental processes of the fungus during infection is poorly understood, and host signals involved in these processes are still unknown. In addition, DNA array analyses have been conducted to identify fungal genes that are expressed during fungal growth in the plant (Kämper et al., 2006). Preliminary data indicate that a set of more than 500 fungal genes is plant regulated. This set includes genes encoding for potential transporters for sugars and amino acids, which is likely to reflect the adaptation of *U. maydis* to the conditions in the plant apoplast. The analysis of these genes may provide important insights into nutrient acquisition during the pathogenic phase.

Recently, the analysis of the available genome sequence revealed that the number of secreted proteins that could serve as effector proteins during the establishment of the fungus/host interface is significantly smaller than in other phytopathogenic fungi (Kämper et al., in press). A very high percentage of the secreted proteins are *U. maydis*-specific at the sequence level and many of the respective genes are arranged in gene clusters of three to 23 genes. Knockout mutants generated for the genes in these clusters revealed a dramatic reduction in virulence and, in one case, mutants were hypervirulent because they caused more pronounced and earlier disease symptoms than the parental wild-type strains. Microscopic observations showed that the mutants arrest at discrete stages of biotrophic growth and many are deficient in tumor formation. Therefore, the secreted proteins are required at different stages of fungal development within plant tissue and could be the long sought-after effectors for establishment of biotrophy. In addition, *U. maydis* seems to be poorly equipped with the cell wall degrading enzymes often used by phytopathogens to attack their hosts (Kämper et al., in press). These
enzymes may be used by *U. maydis* only to penetrate plant cells but not to provide for nutrients during biotrophic growth. This may reflect a specific strategy to minimize host damage during infection, which may help to avoid plant defense responses that are often triggered by cell wall fragments (Mohnen and Hahn, 1993).

One of the unresolved questions in the *U. maydis*/maize interaction is how the fungus evades plant defense responses during its intimate contact with the host. The current hypothesis is that this occurs either by detoxification of reactive oxygen species produced by the host or from active suppression of defense responses. It has also been proposed that *U. maydis* escapes induction of a classical host defense due to its extensive proliferative capacity (Basse, 2005). Changes in transcript levels of maize genes related to metabolism and development have been characterized during *U. maydis* infection (Basse, 2005). Another unresolved question is what are the nutritional requirements of *U. maydis* during its biotrophic phase. Very little is known about the ability of *U. maydis* to utilize nutrients provided by a plant during infection.

### 1.2.2. Disease symptoms caused by *U. maydis*

*U. maydis* is a member of a group of smut fungi that infect a large number of dicotyledonous and monocotyledonous plants, including some of the world’s major cereal crops, reducing yield and causing severe economic losses (Martinez-Espinoza et al., 2002; Agrios, 1988; Christensen, 1963). In general, smut fungi have narrow host range and an individual *Ustilago* species often infect only closely related plant species. For example, *U. maydis* infects only maize (*Zea mays*) and its close relative teosinte (*Euchlena mexicana*). A hallmark of the maize smut disease is the formation of large tumors, which develop on all aerial parts of the plant, leading to stunted growth and significant reduction in crop yield (Christensen, 1963) (Figure 1.1).

Early disease symptoms of infected maize (*Zea mays*) plants are chlorosis (yellowing of tissue), the formation of anthocyanin pigment, and stunted growth (Figure 1.1A and B). Tumor formation is associated with plant cell enlargement and proliferation that takes place later during disease development (Callow and Ling, 1973; Snetselaar and Mims, 1994).
Figure 1.1 Disease symptoms caused by *U. maydis*.

(A) Chlorosis or the yellowing of green leaf tissue is the first sign of infection. (B) Anthocyanin (purple) pigmentation (white arrow) on leaf tissue develops during early stages of infection and tumors also develop on leaves (black arrow). (C) Large tumors near the base of a plant. (D) Tumors produced on developing ears of mature maize plants.
Although *U. maydis* can infect any part of the plant, the ears of mature maize plants are the prime sites of infection (Figure 1.1B, C and D). In maize seedlings, the fungus tends to infect meristematic tissue at the base of the second leaf, which becomes neoplastic (Callow and Ling, 1973). The whole third leaf may become so heavily infected that it fails to unroll. Although infection may be localized to centers of meristematic activity, chlorosis develops in regions of the plant in the absence of fungal hyphae (Callow and Ling, 1973). In the infected cob, the fungus literally replaces the normal kernels with large distorted tumor cells. These tumors, often called galls, are made up of enlarged cells of the infected plant within which the filamentous cells of the fungus proliferate and eventually differentiate into black spores. The galls are at first enclosed in a silvery white membrane. As they mature, the membrane breaks and a black, powdery mass of spores is exposed. The spores give the cob a burned, scorched appearance. The name *Ustilago* comes from the Latin word *ustilare* (to burn). Tumors on the leaves and tassels usually appear as very small galls that eventually become hard and dry.

1.2.3. Early infection stages

As described above, *U. maydis* is a biotrophic pathogen that depends on living plant cells to complete its life cycle (Figure 1.2). Pathogenic development is initiated by the filamentous dikaryon that results from fusion of two compatible haploid cells (1N) (Martinez-Espinoza et al., 1993; Snetselaar and Mims, 1992, 1993). The haploid, non-pathogenic, cells are cigar-shaped, and daughter cells arise by budding (Christensen, 1963; Banuett, 1992). Haploid budding can continue indefinitely as long as nutrients are available. Upon receiving a pheromone signal from a mating partner, the budding haploid cells form conjugation hyphae, which start growing towards each other, and eventually fuse at their tips. The resulting dikaryon (N+N) exhibits filamentous growth. On the plant surface, the dikaryon differentiates and forms appressorium-like structures that allow direct penetration of the cuticle, presumably aided by lytic enzymes (Snetselaar and Mims, 1993).
Figure 1.2 The life cycle of *U. maydis*.

Saprophytic haploid cells (1N) are non-pathogenic and grow by budding. A pathogenic dikaryon (N + N) results from fusion of two compatible haploid cells and has a filamentous morphology. After infection of the plant, tumor formation is induced and fungal filaments differentiate within the tumors to eventually generate teliospores (2N). The diploid spores undergo meiosis to form haploid sporidia. Maintenance of parasitic growth is dependent on the presence of the host (*Zea mays*).
The appressorium in *U. maydis* is not a very prominent structure and differs substantially from the true appressorium (i.e., rounded melanized structures) formed by *M. grisea* and *Colletotrichum graminicola* where entry occurs by mechanical force after build-up of enormous turgor pressure within their appressorium that penetrates plant surface by mechanical force (Bechinger et al., 1999; de Jong et al., 1997). The *U. maydis* appressorium is not melanized and can be seen only as a swelling of the hyphal tip that is not sealed off from the hyphae by a septum prior to penetration (Snetselaar and Mims, 1993). It is not yet clear whether plant entry for *U. maydis* is dependent on lytic enzymes. The fungus produces fibrous material between the appressorium-like swelling and the host cell wall, and this may indicate the production of adhesion matrix that may contain lytic enzymes (Snetselaar and Mims, 1993). The fungal entry into the host may also occur through wounds or through host stomata (Banuett and Herskowitz, 1996). During penetration, the plasma membrane of the host invaginates and surrounds the infection hyphae, shielding the fungus from direct contact with the host cytoplasm (Snetselaar and Mims, 1994; Snetselaar and Mims, 1992; Banuett and Herskowitz, 1996). Following penetration, *U. maydis* grows intracellularly and develops an extensive interaction zone around the infecting hyphae. The establishment of an interaction zone between the plant and fungal membranes is though to be associated with extensive membrane recycling and accumulation of secreted material (Bauer et al., 1997). In contrast, many obligate biotrophic pathogens such as rusts form specialized infection structures (haustoria) that invade host cells without breaching the plant cell plasma membrane; these structures then engage in the acquisition of nutrients and may deliver proteins (Birch et al., 2006; Mendgen and Hahn, 2002). In addition, there is no typical host defense response to *U. maydis* during the early stages of infection and the infected plant tissue remains alive until late in the infection process when fungal proliferation occurs mostly intercellularly (Snetselaar and Mims, 1993).

### 1.2.4. Proliferation and differentiation in the plant host

The dikaryotic filament (hypha) is the pathogenic form in *U. maydis* and consists of elongated cylindrical cells separated by septa containing two nuclei (one from each parent: N+N) (Figure 1.2). In the early stages after penetration, the dikaryotic filament
continues rapid, unbranched growth. Only the growing tip of the filament contains cytoplasm whereas the older compartments lack cytoplasm and collapse (Snetselaar and Mims, 1992; Banuett and Herskowitz, 1996). Growth at this stage of infection is mostly intracellular. Three days post infection (p.i.), the fungus starts growing extensively by producing highly branched hyphae that are filled with cytoplasm (Knowles, 1898; Mills and Kotzé, 1981; Sleumer, 1932; Snetselaar and Mims, 1992). The source that triggers the switch in growth mode is presently unknown, and it corresponds with the beginning of tumor development (approximately five days p.i.). In later stages, hyphal branching increases and occurs at closer intervals. The branched fungal hyphae are surrounded by mucilaginous material. The tips of many hyphae have a lobed appearance, which may be a consequence of the extensive formation of short branches. At this stage, fragmentation of fungal hyphae into segments takes place. Large masses of rounded cells are present as well as cells with other unusual morphologies (e.g., triangular, almond-shaped and peanut-shaped) (Banuett and Herskowitz, 1996). These cells are all embedded in mucilaginous material and many of the rounded cells appear to be in different stages of teliospore maturation. Nuclear fusion (karyogamy) may occur at this stage. Mature teliospores (2N) are surrounded by a yellow-brown cell wall at first, which turns later dark brown or black (melanized) (Banuett and Herskowitz, 1994; Snetselaar and Mims, 1993, 1994, 1992). Thus, the tumors develop a dark colour as masses of teliospores mature inside them. Sporulation is a key event for dissemination of smut disease and teliospores are produced in great numbers (2.5 to 6 billion spores per gram of tumor tissue) during infection (Banuett and Herskowitz, 1996; Christensen, 1963; Ramberg and McLaughlin, 1980). On the plant surface, teliospores germinate and produce short filament (promycelium) forming a basidium in which meiosis takes place. Four nuclei then migrate into individual basidiospore cells (sporidia) that grow by polar budding and exhibit yeast-like morphology. Secondary sporidia produced in nature by budding have been proposed to be the primary infectious agents (Alexopoulos et al., 1996).

Many events in the development of *U. maydis* that are observed *in planta* do not occur in culture. The differences in fungal behaviour *in planta* and in culture have led to conclusion that the plant provides crucial components and/or signaling molecules that trigger different aspects of fungal development. For example, extensive branching of
infection hyphae, formation of cross walls (septa) within hyphae, development of branch primordia resembling clamp connections, random hyphal collapse, prolonged growth of the dikaryon, nuclear fusion in the dikaryon, and teliospore production take place exclusively within host tissue (Banuett and Herskowitz, 1989, Banuett, 1994, 1996; Day and Anagnostakis, 1971; Holliday, 1961; Snetselaar and Mims, 1994.). So far it has not been possible to generate the infectious structures in vitro, which may indicate the need for certain plant signals or surface cues that need to be recognized to trigger this differentiation. The regulatory processes of the developmental program within the host plant leading to teliospore formation are poorly understood, and the signals that may be endogenous or provided by the plant are still unknown. It has been proposed that the stage-specific expression of genes during development is achieved by interplay between repression during saprophytic growth and expression during the specific stage of biotrophic growth in the plant (Basse et al., 2002). The identification of host-derived signals influencing specific stages of fungal development is a challenging aspect of U. maydis virulence that has received relatively little attention.

It has been shown that U. maydis-induced tumor formation is essentially confined to immature tissue at the base of developing leaves and ears (Wenzler and Meins, 1987). Tissue at the base of developing leaves is composed of dividing and expanding meristematic cells (Smith et al., 2001; Sylvester et al., 1990). Tumors result from abnormal division and enlargement of host cells while the hyphae rapidly proliferate between host cells. Within the tumor, the fungal cells are often embedded in parenchymatous, thin-walled host cells, which lack plastids (Callow and Ling, 1973). There is some molecular evidence that U. maydis has the capacity to extend the undifferentiated state of infected tissue (Basse, 2005). U. maydis clearly induces dramatic proliferation of plant tissue and this may occur perhaps either through the production of small inducer molecules, or secretion of fungal effector proteins. It has been shown, for example, that phytohormones known to stimulate plant cell growth (e.g., auxins) are produced by U. maydis in culture (Wolf, 1952). Wolf (1952) observed increased levels of auxin in tumor tissue but clear experimental evidence linking auxin production by U. maydis to tumor development is still lacking. For example, mutants deleted in two genes implicated in the common IAA pathway that catalyzes the
production of auxin from indole-3-acetaldehyde had no defect in pathogenicity (Basse et al., 1996).

1.2.5. Regulation of morphogenesis and pathogenesis in *U. maydis*

As mentioned, *U. maydis* displays a dimorphic switch alternating between budding and filamentous morphology. This morphological switch plays a critical role in pathogenicity because only the filamentous dikaryon can infect the host plant. Therefore dimorphism is a clear component of the disease process. The filamentous phenotype has been used to identify several genes involved in the regulation of morphogenesis, and to examine the link between morphogenesis and pathogenesis in *U. maydis* (Barrett et al., 1993; Brachmann et al., 2001; Durrenberger et al., 1998, 2001; Gold et al., 1994; Lee and Kronstad, 2002). This approach identified two conserved signal transduction pathways, the cyclic AMP/ protein kinase A (PKA) pathway and the Ras/mitogen-activated protein kinase (MAPK) pathway, that are now known to regulate morphological changes during fungal development and to influence the virulence of *U. maydis* (Figure 1.3). The MAPK pathway may also control some aspects of mating in *U. maydis* as described below. It is thought that if there is perception of plant molecules that influence fungal development and disease progression, such signals are likely to be transmitted via the conserved pathways. The cAMP and MAPK pathways related to the yeast pheromone signal transduction cascade also play important roles in morphogenesis and pathogenic development in other pathogenic fungi (D'Souza and Heitman, 2001; Kronstad et al., 1998).

1.2.6. Mating

Fusion of compatible haploid cells is the first event required to generate the pathogenic cell type of *U. maydis*, and this process is initiated by mating-type-specific lipopeptide pheromones that are secreted and perceived by cells of opposite mating type. *U. maydis* belongs to a group of heterothallic fungi that are self-incompatible and only capable of mating with compatible mating partners.
Figure 1.3 Signaling networks in *U. maydis*.
The cAMP/PKA signaling pathway (left) and the Ras/MAPK (pheromone response) signaling pathway (right) regulate mating, morphogenesis, and virulence. AC = adenyl cyclase; PKA-R = the regulatory subunit of PKA; PKA-C = catalytic subunit of PKA.
These fungi have two or more mating types and the sexual reproduction can occur only when individuals of different mating types interact. In *U. maydis*, the sexual life cycle is governed by a tetrapolar mating system consisting of the $a$ and $b$ mating type loci (Kronstad and Staben, 1997). Haploid cells are able to fuse and form a stable dikaryon only if they carry genes with different specificities at both the $a$ and $b$ mating-type loci. Cell recognition, conjugation tube formation and cell fusion are controlled by the $a$ locus having two alleles $a1$ and $a2$, which encode pheromones and pheromone receptors similar to those in *S. cerevisiae* (Banuett and Herskowitz, 1989; Böller et al., 1992; Puhalla, 1969; Rowell, 1955; Snetselaar et al., 1996; Spellig et al., 1994). Upon pheromone stimulation, cells arrest budding growth and start the formation of conjugation tubes (Snetselaar and Mims, 1992; Spellig et al., 1994), which grow towards each other following a pheromone gradient and which eventually fuse (Snetselaar et al., 1996). Once cell fusion has taken place, heterozygosity at the $b$ locus is required for the production of a stable dikaryon and for pathogenicity. This locus is multi-allelic and encodes two homeodomain-containing proteins (bE and bW). After cell fusion, these proteins interact to produce a heterodimeric complex only when the proteins are encoded by different alleles (Gillissen et al., 1992; Kamper et al., 1995; Yee and Kronstad, 1993). The b protein heterodimer is thought to act as a master switch to initiate filamentous growth and subsequent pathogenic development by regulating the transcription of a set of target genes that directly or indirectly govern morphogenesis and pathogenicity, and completion of life cycle (Gillissen et al., 1992; Kamper et al., 1995; Böller et al., 1995; Kronstad and Leong, 1989; Schulz et al., 1990). In addition, the b protein heterodimer appears to repress the expression of pheromones and pheromone receptors (Laity et al., 1995). In culture, sexually compatible haploid strains are able to mate and produce dikaryotic hyphae (Figure 1.4). The combination of the compatible strains is infectious when inoculated into a host plant. In contrast, the dikaryotic hyphae are short-lived and eventually die when left on artificial medium (Holliday, 1974; Puhalla, 1968).
Figure 1.4 A dikaryotic filament produced during the mating reaction between haploid cells of *U. maydis*.

Upon pheromone signal exchange, haploid cells of opposite mating type form conjugation tubes and these eventually fuse to produce infectious dikaryotic filaments. Compatible haploid sporidia shown here were grown on fatty acid-containing medium (palmitate) to observe mating reaction. The image was taken using differential interference contrast optics (DIC).
1.2.7. MAP kinase signaling

Upon the exchange of pheromones between the compatible haploid cells, the pheromones bind to cognate seven-transmembrane domain receptors (Pra1/2) and activate downstream signaling cascades, such as a MAPK cascade. This leads to expression of a large number of genes mediated by the pheromone response factor (Prfl) that binds to pheromone response elements (PRE) present in promoter regions (e.g., in the \textit{a} and \textit{b} mating type genes) (Hartmann et al., 1996); (Urban et al., 1996). Mutants defective in Prfl are sterile because of their inability to perceive and produce pheromones. The main role of Prfl seems to be the induction of mating type genes. Finally, pheromones and pheromone receptors are not required for filamentous growth during the biotrophic phase. Therefore, it has been argued that signals from the host environment act as alternative inducers of the MAPK pathway (Banuett and Herskowitz, 1996).

The MAPK cascade consists of the MAPK kinase kinase Ubc4/ Kpp4 (Andrews et al., 2000; Muller et al., 2003), the MAPK kinase Fuz7/Ubc5 (Andrews et al., 2000; Banuett and Herskowitz, 1994) and the MAPK Ubc3/Kpp2 (Mayorga and Gold, 1999); (Muller et al., 2003). Mutations in any of the MAPK pathway components result in faulty pheromone signaling (Hartmann et al., 1996). In addition, Ubc3 is required for tumor induction (Mayorga and Gold, 1999) and Ubc2 is required for full symptom development during host infection (Mayorga and Gold, 2001). The \textit{ubc2}, \textit{ubc3}, \textit{ubc4} and \textit{fuz7} genes were all identified using a morphological screen in which complementation of suppressor mutations resulted in the restoration of filamentous growth to yeast-like suppressors of the filamentous growth of a mutant deficient in the gene encoding for adenyllyl cyclase (\textit{uacl}; (Andrews et al., 2000; Gold et al., 1994;Mayorga and Gold, 2001). The \textit{ubc4}, \textit{fuz7} and \textit{ubc3} genes were also isolated in independent studies and called \textit{kpp4}, \textit{ubc5} and \textit{kpp2}, respectively (Muller et al., 1999; Andrews et al., 2000; Muller et al., 2003). The MAPK module has been shown to also regulate \textit{b}-dependent filament formation (Fuz7), cuticle penetration (Brachmann et al., 2003), appressorium-like structure formation (Muller et al., 2003) and cell cycle regulation (Garrido et al., 2004). This is in addition to the regulation of the transcriptional response to pheromone, conjugation tube formation and cell fusion (Ubc4, Fuz7 and Ubc3).
Complementation of a suppressor mutation of the adrl mutant (defective in the catalytic subunit of PKA) identified the ras2 gene, which encodes a member of the Ras family of small GTP-binding proteins (Lee and Kronstad, 2002). Ras2 has been shown to influence the MAPK pathway and to regulate morphogenesis, pathogenesis and mating. *U. maydis* possesses a second Ras protein, Ras1, which is proposed to effect the cAMP pathway (Muller et al., 2003).

1.2.8. cAMP signaling pathway

The cAMP/PKA signaling pathway is a key regulator of the dimorphic switch and pathogenic development in *U. maydis*. From the analysis of mutants defective in cAMP/PKA signaling, it is apparent that a regulated cAMP pathway is essential for penetration, proliferation in plant tissue, tumor induction and teliospore production. An active cAMP pathway appears to be crucial for entry into the plant because strains deficient in either the Gα subunit of a heterotrimeric G protein (Gpa3; Regenfelder et al., 1997), adenylyl cyclase (Uacl; Barrett et al., 1993), or the catalytic subunit of PKA (Adrl; Durrenberger et al., 1998) fail to produce any disease symptoms (Durrenberger et al., 1998; Regenfelder et al., 1997). Mutants lacking the regulatory subunit of PKA (Ubc1) display constitutive PKA activity and proliferate in plant tissue, but fail to produce tumors (Gold et al., 1997). Mutants with a constitutively active gpa3 allele also show reduced proliferation and do not produce teliospores, but still induce tumors (Kruger et al., 1998). Strains deleted in hgll produce tumors but spore formation is abolished (Durrenberger et al., 2001). Hgll is a protein with unknown function that appears to be a direct target of PKA, and is thought to act as a negative regulator of budding growth and pigment production. It may also be an activator of filamentous growth and teliospore formation (Durrenberger et al., 2001).

The initial discovery of a role for the cAMP pathway in *U. maydis* came from the identification of the uacl gene for adenylyl cyclase in a genetic screen using ultraviolet light (UV) to mutagenize cells and isolate constitutively filamentous mutants (Barrett et al., 1993; Gold et al., 1994). Subsequently, ubcl was identified as a suppressor of the constitutive filamentous phenotype of the uacl mutants (Gold et al., 1997). The ubcl
mutants exhibit a multi-budding phenotype in which daughter cells remain attached to mother cells to form rosette-like clusters (Gold et al., 1994). The hglI gene was isolated in a suppressor mutant screen that was designed to identify downstream targets of the cAMP pathway. The hglI gene was identified through a complementation of one suppressor mutation that resulted in the restoration of filamentous growth to a yeast-like adrl suppressor mutant (Durrenberger et al., 2001). Two genes encoding PKA catalytic subunits in U. maydis (adrl and ukaI) were cloned by PCR amplification using degenerate primers. Mutants defective in adrl exhibit a constitutive filamentous phenotype and are avirulent. In contrast, the deletion of ukaI had only a minor influence on morphogenesis and virulence. That is, the ukaI mutants are predominantly yeast-like and are still able to cause disease in maize.

In general, all conditions that lower cAMP levels, such as mutations in gpa3 or uac1, or conditions that reflect this situation in terms of PKA activity (mutation of adrl) result in filamentous growth (Durrenberger et al., 1998; Gold et al., 1994; Regenfelder et al., 1997). Contrary to this, conditions that lead to constitutive activation of the pathway, and therefore high PKA activity, such as deletion of ubc1 encoding the regulatory subunit of the PKA, lead to a budding phenotype (Gold et al., 1997). Taken together, these findings show that cAMP levels are responsible for the dimorphic switch, as well as virulence and cytokinesis, and temporal regulation of the cAMP pathway appears to be critical for the completion of the life cycle.

1.2.9. Crosstalk between the cAMP and MAPK signaling pathways

There appears to be an intimate connection between cAMP and MAP kinase signaling in U. maydis (Figure 1.3). When genetic screens were conducted to identify additional players in signaling pathways using the filamentous phenotype of uac1 or adrl mutants (i.e., defective in cAMP signaling) to identify suppressors, these turned out to be mutations in all four components of the pheromone MAP kinase cascade and ras2. (Andrews et al., 2000; Lee and Kronstad, 2002; Mayorga and Gold, 1998, 1999, 2001). In light of these results, there may be other signals that lead to activation of the MAP kinase cascade other than pheromones. This result is the foundation for naming the components of the MAP kinase cascade as ubc genes for Ustilago bypass of cyclase. The
general view to emerge from these studies is that the components from the MAP kinase pathway induce filamentation while the cAMP/PKA pathway represses this morphological transition. At present, it is not entirely clear at which level(s) these opposing effects operate. Perhaps co-regulation of the cAMP and MAPK signaling networks is involved in sensing specific plant signals that trigger discrete stages of pathogenic development.

The cAMP signaling pathway is also necessary for pheromone response, and putative phosphorylation sites have been predicted for both MAPK and PKA in the polypeptide sequence of the Prf1 transcription factor (Hartmann et al., 1996). Furthermore, signaling via the cAMP pathway appears to influence pheromone expression via Prf1 at both the transcriptional and translational levels (Hartmann et al., 1996). In addition, the gpa3 mutants were unable to induce pheromone expression when mixed with compatible strains (Regenfelder et al., 1997). Kruger et al. (1998) showed an increased pheromone gene expression in ubc1 mutants and wild-type cells grown in the presence of exogenous cAMP. Thus, an interplay between cAMP levels and MAPK signaling may influence pheromone signaling.

1.3. Lipid signaling in fungal pathogens

The characterization of the cAMP and MAPK pathways in *U. maydis* provides a framework and a set of mutants for further investigation of the role of signaling functions in fungal phytopathogenesis. This includes the search for environmental, nutritional or host factors that trigger morphological transitions as a key aspect of pathogenesis. In this context, a role for lipids in morphogenesis and development has been identified in a limited number of fungi, including *U. maydis* as described in this thesis (Chapter 2 and Klose et al., 2004). Therefore, the following sections provide background information to summarize what is known about connections between lipids, signaling and pathogenesis in fungi.
1.3.1. Lipids and early stages of fungal development

Plant surface lipids (waxes) have been reported to provide signals in plant-fungus interactions and are thought to induce pathogenic development in fungi (Macko, 1981; Podila et al., 1993). For example, cutin monomers of avocado contain inducers and inhibitors of germination and appressorium formation required in the initial stages of infection by *C. gloeosporioides* (Kolattukudy et al., 1995; Podila et al., 1993). The balance between these activities may be responsible for the selective signaling by the host wax as a component of host recognition. Appressorium formation was most strongly initiated in response to long-chain fatty alcohols (C24 or longer) present in the host wax, and waxes from non-host plants failed to induce appressorium formation in *C. gloeosporioides*. This suggests that the effect of the surface lipids vary with fungal species. Fungal storage lipids also appear to play an important role in the early developmental processes of pathogenic fungi, such as the formation of appressoria (Thines et al., 2000). Specifically, it has been shown that PKA signaling is involved in the mobilization of the storage compounds, such as lipids and glycogen, during the production of infectious structures that are critical for penetration of the host cells in the plant pathogen *M. grisea* (Thines et al., 2000).

1.3.2. Lipids in development and sporogenesis in fungi

Lipids have been shown to regulate reproductive development in several fungi. Linoleic acid in particular has a sporogenic effect in *Alternaria, Aspergillus, Neurospora* and *Sclerotinia* (reviewed in Calvo et al., 2001). In general, unsaturated long-chain fatty acids (LCFA; e.g., oleic and linoleic acid) and their derivatives (e.g., oxygenated linoleic and oleic acids called oxylipins) are reported to be important for the sexual development of filamentous fungi. In *Aspergillus* spp., fatty acid stimulation of sporulation is dependent on fatty acid chain length and the presence of double bonds, and oleic and linoleic acids have the greatest stimulatory effect (Calvo et al., 2001). In *Neurospora crassa*, extensive changes in fatty acid metabolism correlate with several events during sexual development (Goodrich-Tanrikulu et al., 1998). In this case, the availability of oleic and linoleic acids dictates the fate of development in *N. crassa*. Specifically, oleic
acid is the predominant fatty acid found in sexual structures, whereas linoleic acid is the predominant fatty acid in asexual tissues. One of the first extracellular signals described to regulate sexual and asexual spore development in filamentous fungi was psi (precocious sexual inducer) factor characterized in *Aspergillus nidulans* (Calvo et al., 2001). Psi factors are oleic, linoleic and linolenic acid-derived oxylipins that act as signaling molecules to influence growth and spore development in *A. nidulans* (Calvo et al., 2001; Champe et al., 1987; Mazur et al., 1990). Certain species of oxylipins are generated from enzymatic reactions catalyzed by lipoxygenase or dioxygenase enzymes in fungi (Herman, 1998; Noverr et al., 2003). In *A. nidulans*, deletion of genes encoding oxylipin-generating dioxygenases (*ppoA*, *ppoB* and *ppoC*) influenced the expression of transcription factors required for meiotic and mitotic sporulation processes (Tsitsigiannis et al., 2004a; Tsitsigiannis et al., 2005b; Tsitsigiannis et al., 2004b). Similar oxylipins are also produced by other fungi, such as *Gaeumannomyces graminis*, *Fusarium oxysporum* and *Saccharomycopsis* spp. (Brodowsky et al., 1992; Nakayama et al., 1996; Sebolai et al., 2005; Su et al., 1995; Su and Oliw, 1996). For example, in *G. graminis*, the linoleate diol synthase (LDS) has been characterized that produces psiBα (8-hydroxylinoleic acid or 8-HODE) that has an effect on development (Brodowsky et al., 1992; Su et al., 1995; Su and Oliw, 1996). Recently, the cAMP-regulated *sspl* gene encoding a protein abundantly expressed in mature teliospores of *U. maydis* was identified and it shares similarity with LDS and prostaglandin G/H synthases (cyclooxygenases) from mammals (Huber et al., 2002). It is thought that Ssp1 plays a role in the mobilization of storage lipids, probably as part of teliospore maturation in *U. maydis*. The fungal pathogens of humans, *Candida albicans* and *Cryptococcus neoformans*, are able to convert exogenously supplied as well as endogenously produced arachidonic acid into bioactive prostaglandins (modified lipids: oxygenated unsaturated cyclic fatty acids) which strongly enhance both cell viability and filamentation capacity (Noverr et al., 2001, 2002).
1.3.3. Cellular lipids and fungal dimorphism

The human fungal pathogen *C. albicans* is a well-characterized model for fungal dimorphism and some information is available on the role of lipids. For example, differences in membrane composition/properties were observed between yeast and mycelial forms of the fungus. A comparison of lipids of purified plasma membranes revealed that the composition of the plasma membrane of *C. albicans* resembles that of other eukaryotes (Marriott, 1975). An increase in linoleic acid in all membrane phospholipids correlated with elongation of germ tubes during conversion of yeast to mycelium form (Yamada, 1986). Higher levels of polyunsaturated fatty acids were observed in mycelial lipids compared to yeast lipids (Yano et al., 1982). Overall, the mycelial lipids are poorer in sterols than the yeast lipids, but are richer in complex lipids that contain sterols such as sterylglycosides and esterified steryl glycosides (Ghannoum et al., 1986). The observed fluctuation of fatty acids, sterols and sterol-containing complex lipids in the yeast and mycelial forms suggest a possible role of cellular lipids in the dimorphic behaviour of *C. albicans*.

1.4. Lipid metabolism in fungal pathogenesis

To understand fungal infection, it is important to understand the requirements of pathogenic fungi to access various nutrients to optimize morphological and metabolic differentiation while growing in vivo. Recent findings on pathogen nutritional requirements and the mechanisms by which they acquire nutrients from their host during infection indicate that primary metabolism (as expected) plays a significant role in virulence and disease development (Both et al., 2005; Lorenz et al., 2004; and reviewed in (Solomon et al., 2003). In particular, several genome-wide studies of gene expression patterns reveal important roles for lipid metabolism during fungal infections. For example, microarray data reveal a fascinating pattern of coordinate regulation of genes encoding enzymes involved in primary metabolism at different stages in the life cycle of a plant pathogen *B. graminis* (Both et al., 2005). These include enzymes involved in lipid catabolism that are highly expressed in early stages of infection but that show decreased expression in later stages. Storage lipids in conidia of *B. graminis* are used
during penetration and colonization of the host plant and then again later in the life cycle when new conidia are formed. Previously, expressed sequence tags (ESTs) and serial analysis of gene expression (SAGE) have also shown that lipid catabolism is important throughout the germination and penetration stages of infection of *B. graminis* (Thomas et al., 2002; Thomas et al., 2001). Recently, the expression patterns identified from a microarray experiment suggest a cell-specific difference in nutrient acquisition and cell metabolism in *U. maydis* (Babu et al., 2005). Specifically, the peroxisomal multifunctional enzyme (designated by the authors as *fox2*) is upregulated during the filamentous growth compared to budding cell growth in culture. Also, in the microarray experiment *fox2* is upregulated in both dikaryotic and diploid state of the filamentous cells. Note that this gene is the subject of chapter 3 of this thesis and is designated as *mfe2* in this work. Furthermore, transcriptome profiles of the human pathogenic fungus *C. albicans* revealed regulation of metabolic pathways involved in utilization of alternative carbon sources upon phagocytosis by macrophages, specifically by reprogramming metabolism to produce glucose (Lorenz et al., 2004). The data suggest that the acetyl coenzyme A (acetyl-CoA) derived from breakdown of fatty acids via β-oxidation is used via the glyoxylate cycle to produce glucose. The glyoxylate cycle has been shown to play a role in virulence of many pathogens, including the fungal plant pathogens *Leptosphaeria maculans* (Idnurm and Howlett, 2002), *M. grisea* (Wang et al., 2003) and *Stagonospora nodorum* (Solomon et al., 2004), the bacterial plant pathogen *Rhodococcus fascians* (Vereecke et al., 2002), and the human pathogens *Mycobacterium tuberculosis* (McKinney et al., 2000) and *C. albicans* (Lorenz and Fink, 2001). Furthermore, peroxisomal metabolic function is essential for plant infection during the penetration stage in the fungal pathogen *Colletotrichum lagenarium* (Kimura et al., 2001). Specifically, the generation of a targeted mutation in the gene *PEX6* required for peroxisomal biogenesis, where enzymes for β-oxidation of fatty acids reside, impairs peroxisomal metabolism and leads to the loss of pathogenicity. Taken together, these studies suggest that lipid metabolism may be important during plant-fungus interactions that lead to a successful infection.

Different aspects of lipid metabolism may be important for fungal pathogens during specific stages of infection. In the initial stages before penetration, the fungus
depends on stored sources of carbon, which possibly include glycogen, trehalose, sugar alcohols and lipids (Jennings and Lysek, 1996; Thines et al., 2000; Weber et al., 2001). Recent analyses show that lipid catabolism is critical during early stages of infection, such as germination, production of infectious structures (i.e., appressoria formation) and penetration (Bowyer et al., 2000; Kimura et al., 2001; Thines et al., 2000; Weber et al., 2001). During later stages of fungal development in the plant, when fungi extensively proliferate within host tissues, lipolysis may not play as important of a role (reviewed in Solomon et al., 2003). After penetration, sugars from the host (e.g., sucrose) are available and may become the main energy source. It has been proposed that fungal metabolism throughout the infection process can be divided into three phases, where the first phase involves lipid catabolism during germination and penetration and the second phase involves glycolysis during invasion of host tissue (reviewed in Solomon et al., 2003). The third phase occurs late in infection during sporogenesis, when the host tissues are increasingly incapacitated by the pathogen and the fungus begins to produce spores for dissemination. At this late stage, nutrients are likely to be depleted and this could lead to starvation of the pathogen as one aspect for signaling sporulation (Solomon et al., 2003). However, nutrient availability during this stage has not yet been studied.

It has been shown that utilization of plant host lipids is of great importance to some fungal pathogens. In the bunt fungi (*Tilletia* spp.), which are closely related to *U. maydis*, host plant lipids were shown to be of primary importance in the metabolism of hyphae and spores. Histochemical studies of *Tilletia carries* (common bunt of wheat) developing in young infected wheat seeds revealed that lipids were the first host compounds utilized by pathogenic hyphae that produced teliospores (Grove, 1973). *A. flavus* was also shown to preferentially target lipid bodies (i.e., stored plant lipids) rather than starch for degradation during infection when grown in living corn kernels (Smart et al., 1990). However, in corn-kernel stimulating medium, *A. flavus* simultaneously hydrolyzed starch and lipids (Mellon et al., 2005), suggesting that preference for lipids may be influenced by the nutritional composition of a substrate. A plant fungal pathogen, *Plasmodiophora brassicae*, utilizes lipids as temporary carbon sources synthesized from precursors extracted from the host plant, and accumulates a large number of lipid bodies after it enters its *Brassica* host plant cytoplasm during infection (Williams et al., 1968;
Keen and Williams, 1969). Furthermore, a variety of studies indicate that fungal pathogens can alter host lipid content or metabolism during infection. For example, changes in total fatty acids composition of kidney bean plant (*Phaseolus vulgaris*) were reported at different stages of infection with rust fungus *Uromyces phaseoli* (Schnipper and Mirocha, 1970). Schmidt (1932) provided microscopic evidence of lipid accumulation adjacent to infection hyphae during infection of sugar-beet leaves. Taken together, these studies indicate that the utilization of lipids as alternative carbon sources plays an important role in microbial pathogenesis.

Lipids also play a role in fungal plant pathogenesis because of their involvement in host defense. Unsaturated fatty acids have lately emerged as important players in diverse biological processes in plants (Kachroo et al., 2001, 2003; Laxalt and Munnik, 2002; Lee et al., 1997; Li et al., 2003; Maldonado et al., 2002; Piffanelli and Murphy, 1999; Ryu and Wang, 1998; Shanklin and Cahoon, 1998; Weber, 2002). They serve as substrates for biosynthesis of oxidized lipids and also regulate the activity of enzymes involved in the generation of signal molecules to activate plant defense responses. Specifically, fatty acids play an important role in modulating signaling between salicylic acid (SA)- and jasmonic acid (JA)-dependent defense pathways against pathogens (Kachroo et al., 2003). Biotrophic fungi may escape plant defense mechanisms by bypassing or repressing the activation of programmed cell death initiated by jasmonic acid signaling and defense responses by the salicylic acid-dependent pathway. Recent studies have uncovered an important role for lipids also in the activation of systemic acquired resistance (SAR) (Maldonado et al., 2002; Nandi et al., 2004). Interestingly, the β-oxidation pathway in plants has been proposed to be involved in the specific modification of fatty acid signaling molecules of the octadecanoic pathway during the formation of jasmonic acid from linolenic acid (Wasternack and Parthier, 1887). Microarray analysis revealed induction of β-oxidation genes in the model plant *Arabidopsis thaliana*, in local and also in distal tissue, during infection with *Alternaria brassicicola* (Schenk et al., 2000). Therefore, it has been hypothesized that β-oxidation in plants may be playing a role in mediating plant-pathogen interactions.
1.4.1. β-oxidation of fatty acids

Depending on the metabolic demands of a cell, fatty acids are either converted to triglycerides and membrane phospholipids, or oxidized for energy production. The cellular fatty acid degradation into acetyl-CoA occurs via the β-oxidation pathway. The β-oxidation of fatty acids is a metabolic process providing electrons to the respiratory chain and thus energy for a cell. It is a complex process occurring inside mitochondria or peroxisomes and must be carefully regulated depending on the other sources of energy (i.e., carbohydrate and amino acid catabolism). The consequences of dysfunction in β-oxidation can cause severe health problems in humans. Thus understanding the basic mechanisms of the process is of great relevance and the β-oxidation systems in animals have been well characterized, both biochemically and molecularly (reviewed in Kunau et al., 1995; Eaton et al., 1996; Hashimoto, 1996; Hiltunen et al., 1996; Mannaerts and van Veldhoven, 1996).

The process of β-oxidation is common to all eukaryotic and prokaryotic organisms. There are two major systems of β-oxidation, mitochondrial and peroxisomal. Plants and fungi are able to completely degrade fatty acids within their peroxisomes (Kunau et al., 1988; Tolbert, 1981), while animals require an additional mitochondrial β-oxidation system because the peroxisomal system seems to function only to provide a chain-shortening activity (Kunau et al., 1988; Lazarow and De Duve, 1976; Mannaerts and Debeer, 1982; Tolbert, 1981). The discovery of a peroxisomal system of β-oxidation was first made in plants (Cooper and Beevers, 1969).

β-oxidation results in the complete degradation of fatty acids by the sequential removal of 2 carbon units in each fatty acid oxidation cycle, resulting in the formation of acetyl-CoA (Figure 1.5). Fatty acids inside the cell must be activated initially by fatty acyl-CoA synthetase, which ligates CoA to a free fatty acid (Black and DiRusso, 2003). There are four individual enzymatic reactions of β-oxidation, each catalyzed by a separate enzyme: acyl-CoA dehydrogenase in mitochondria and acyl-CoA oxidase in peroxisomes, enoyl-CoA hydratase, hydroxyacyl-CoA dehydrogenase, and acyl-CoA acetyltransferase (thiolase).
Figure 1.5 β-oxidation of fatty acids.

Fatty acids are oxidized via the β-oxidation process in which fatty acid chains are degraded to acetyl CoA by sequential removal of two carbon units in each fatty acid oxidation cycle. There are four individual reactions of β-oxidation and each is catalyzed by a separate enzyme: (i) acyl CoA-dehydrogenase in mitochondria or acyl-CoA oxidase in peroxisomes, (ii) enoyl-CoA hydratase, (iii) hydroxyacyl-CoA dehydrogenase, and (iv) acyl-CoA transferase (thiolase).
Additional enzymes are needed for complete oxidation of unsaturated and odd-carbon fatty acids: enoyl-CoA isomerase, propionyl-CoA carboxylase, methylmalonyl-CoA racemase, and methylmalonyl-CoA mutase.

1.4.1.1. β-oxidation systems in mammalian cells

There are major differences among different β-oxidation systems including substrate specificities, subcellular compartmentalization, and enzyme architecture. This is well represented in the variety of β-oxidation strategies found in mammalian systems (Figure 1.6). β-oxidation occurs in both mitochondria and peroxisomes. There are two mitochondrial β-oxidation systems. One is a membrane-associated pathway that shortens long-chain fatty acids for the second, soluble, short-chain specific pathway (Kunau et al., 1995). These pathways also differ in enzyme architecture. The short-chain pathway consists of four individual enzymes, while in the long-chain pathways the second to fourth steps are catalyzed by a trifunctional enzyme (Uchida et al., 1992). Electrons removed during mitochondrial β-oxidation are passed to oxygen via the electron transport chain, contributing to ATP formation (Frerman, 1988). There are also two different β-oxidation pathways in peroxisomes of mammalian cells (reviewed in Hashimoto, 2000; Wanders et al., 2001). Two separate multifunctional enzymes with different substrate specificities and stereochemistry catalyze the second and third steps in each pathway. The mammalian multifunctional proteins prefer medium-, long- and very long-chain acyl-CoA esters (Jiang et al., 1996; Malila et al., 1993). Electrons removed during peroxisomal β-oxidation are transferred to oxygen, generating H$_2$O$_2$, which requires catalase activity for detoxification. In addition, the mammalian peroxisomal β-oxidation process has two different acyl-CoA oxidases that catalyze the first step (Casteels et al., 1990) and that exhibit different substrate specificities. Mammalian oxidases preferentially react with medium-, long-, and very long-chain acyl-CoA esters and cannot accept short-chain substrates such as butyryl-CoA (C4) (Vanhove et al., 1991).
In peroxisomes, oxidases differ in their substrate specificities. Multifunctional enzymes possess enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase activities where type 1 enzymes (MFE1) catalyze oxidation of branched- and straight-chain fatty acids and type 2 enzymes (MFE2) catalyze only straight-chain fatty acids. In mitochondria, two independent pathways have been characterized: the first pathway degrades medium- and short-chain fatty acids in mitochondrial matrix via four individual enzymatic steps, and the second pathway consisting of acyl-CoA dehydrogenases and trifunctional protein (TFP) degrades long-chain fatty acids.
Two peroxisomal 3-ketoacyl-CoA thiolases are known to catalyze the third step and these include the classical thiolase (Miyazawa et al., 1981) and a thiolase that is part of the sterol carrier protein SCPx (Seedorf et al., 1994). Both thiolases have overlapping activities.

1.4.1.2. β-oxidation in *Saccharomyces cerevisiae*

β-oxidation in lower eukaryotes and prokaryotes has been found to occur via pathways similar to those described above for mammals. It has been shown that β-oxidation occurs exclusively in the peroxisomes of the yeasts such as *S. cerevisiae*, *Yarrowia lipolytica*, and *C. tropicalis* (Hiltunen et al., 1992; Kunau et al., 1988; Kurihara et al., 1992; Smith et al., 2000). In general, the peroxisomes in the yeast fungi contain the full complement of enzymatic machinery to completely degrade fatty acids. These findings led to the idea that fungi lack mitochondrial β-oxidation. However, recently Maggio-Hall and Keller (2004) have shown that the filamentous fungus *A. nidulans* possesses both mitochondrial and peroxisomal β-oxidation systems.

*S. cerevisiae* is able to grow on fatty acids as a sole carbon source. The growth on fatty acids induces transcriptional up-regulation of genes encoding enzymes of the β-oxidation pathway (Veenhuis et al., 1987). This response is additionally accompanied by a remarkable proliferation of the peroxisomal compartment (De Duve and Baudhuin, 1966). *S. cerevisiae* is able to degrade both saturated and unsaturated fatty acids.

Enzymes involved in β-oxidation are synthesized in the cytoplasm and then imported into the matrix of the peroxisome in an evolutionary conserved manner that is dependent on a subset of peroxins and on *cis*-acting peroxisomal targeting signals (PTTs). There are three PTTs found in the peroxisomal proteins in yeast: PTT1 (a carboxy-terminal tripeptide of the sequence SKL or a conserved variant of this sequence), PTT2 (found at or near the amino termini), and PTT3 (conserved sequence repeats found anywhere within the protein sequence) (de Hoop and Ab, 1992; Kamiryo et al., 1989; Purdue and Lazarow, 1994; Small et al., 1988).
1.4.1.3. **Induction of the peroxisomal β-oxidation system**

In *S. cerevisiae*, the transcription factors Pip2 and Oaf1 regulate the induction of genes encoding peroxisomal proteins (Karpichev and Small, 1998), particularly the genes involved in β-oxidation of fatty acids. The promoter sequences of these genes contain a positive cis-acting element called an oleate response element (ORE) that mediates the induction of these genes by fatty acids in the medium. It has been demonstrated that Pip2 and Oaf1 interact with each other and form a heterodimer that binds ORE (Karpichev and Small, 1998; Rottensteiner et al., 1996); this protein is required for fatty acid-induced peroxisomal proliferation and for regulating the expression of proteins required for β-oxidation of fatty acids.

1.4.1.4. **Substrates and enzymatic reactions of peroxisomal β-oxidation**

*S. cerevisiae* is able use a variety of different saturated and unsaturated long-chain fatty acids as a sole carbon source for growth (van Roermund et al., 2003). These include palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). Saturated medium-chain fatty acids, including lauric acid (C12:0) are also substrates for β-oxidation (i.e., can be oxidized) but appear lethal to yeast when used as a sole carbon source, although myristic acid (C14:0) can be used as a sole carbon source. Short-chain fatty acids such as octanoic acid (C8:0) are also lethal. In addition, very long-chain fatty acids (VLCFA), such as arachidonic acid (C20:4), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) can serve as a sole carbon source. Although some VLCFA (i.e., C24:0 and C26:0) can be oxidized by *S. cerevisiae*, they cannot be used as a sole carbon source for growth.

The process of β-oxidation of fatty acids in yeast follows the same set of enzymatic reactions as found in mitochondria and peroxisomes from higher eukaryotes. The first step involves the introduction of a double bond between the alpha and beta carbon atoms of activated fatty acids catalyzed by acyl-CoA oxidase, which is encoded only by a single gene (*FOX1* or *POX1*) in *S. cerevisiae* (Dmochowska et al., 1990). Fox1 is the ortholog of the human acyl-CoA oxidase 1 (Luo et al., 1996). The second and third reactions in β-oxidation are catalyzed by a multifunctional enzyme (Fox2) with both 2-enoyl-CoA hydratase activity and 3-hydroxyacyl-CoA dehydrogenase activity (Hiltunen
et al., 1992). The final step of peroxisomal β-oxidation is catalyzed by a single 3-ketoacyl-CoA thiolase (Fox3 or Pot1) (Igual et al., 1991). This enzyme catalyzes a thiolytic cleavage of 3-ketoacyl-CoA esters into a C2-shortened acyl-CoA and acetyl-CoA. The expression of the β-oxidation enzymes is strongly induced by fatty acids (Einerhand et al., 1991).

*S. cerevisiae* can oxidize both cis- and trans- unsaturated fatty acids. Saturated and unsaturated fatty acids with a trans double bond at the even-numbered position are direct substrates for the classical β-oxidation pathway. However, unsaturated fatty acids with trans and cis double bonds at odd-numbered positions or cis double bonds at even positions require the participation of additional enzymes during oxidation (reviewed in Trotter, 2001).

### 1.4.1.5. Fatty acid transport

Import of fatty acids from the growth medium involves passive diffusion across the cell membrane in combination with an active, protein-mediated component that includes proteins of the fatty acid transport protein (FATP) family. The protein encoded by the yeast gene *FAT1*, a homologue of the mammalian adipocyte fatty acid transporter protein FATP, was proposed to function as a fatty acid transporter protein (Faergeman et al., 1997; Schaffer and Lodish, 1994). Five additional genes (*FAA1-4* and *FAT2*) encoding proteins with similarity to acyl-CoA synthetases have been described (Johnson et al., 1994). The Faa1 and Faa4 are present in cytosol and are required for activation of imported exogenous fatty acids (Duronio et al., 1992; Johnson et al., 1994). FAA2 and FAA3 encode for acyl-CoA synthetases that can only access fatty acids synthesized within the cell (Knoll et al., 1993). There are two different routes for transport of the substrates for β-oxidation into the peroxisome (Hettema et al., 1996). First, fatty acids such as MCFA enter peroxisomes as free fatty acids and are subsequently activated by Faa2, the peroxisomal acyl-CoA synthetase. Secondly, fatty acids can enter peroxisomes as activated CoA esters via Pxa1-Pxa2 proteins (which comprise the two halves of an ABC transporter) (Hettema et al., 1996; Shani et al., 1995). The activation occurs outside peroxisomes catalyzed by extra-peroxisomal synthetases. This pathway is required to transport activated LCFA (Hettema et al., 1996). β-oxidation products can be transported
from peroxisomes to mitochondria via two different pathways (van Roermund et al., 1995). One is via the glyoxylate cycle to enter gluconeogenesis for production of glucose, and another via the carnitine transport pathway to enter mitochondria and subsequently the TCA cycle to produce energy (van Roermund et al., 1995).

1.4.2. Hydrolytic enzymes: Phospholipases and Lipases

In the context of lipid metabolism, secreted extracellular lipolytic enzymes have been documented as important virulence factors in some fungal pathogens (Chen et al., 1997; Hube, 1998; Ibrahim et al., 1995; Leidich et al., 1998; Saffer et al., 1989; Walker et al., 1983). Pathogenic fungi are known to secrete various hydrolytic enzymes such as proteinases, phospholipases and lipases that are involved in various signaling events regulated by integrated cellular networks. These enzymes are thought to contribute to fungal pathogenesis by disrupting host cell walls, membranes, and extracellular matrices assisting in cell penetration upon infection and tissue invasion.

1.4.2.1. Phospholipases

Phospholipases are a group of enzymes that share the ability to hydrolyze one or more ester linkages in glycerophospholipids. Different phospholipases have the ability to cleave a specific ester bond, therefore they were named with letters A, B, C, and D to indicate the specific bond targeted in the phospholipid molecule (Figure 5.1). Phospholipases are often found in association with membranes and may play a role in intracellular signaling pathways. Several plant and human pathogenic fungi produce phospholipid-hydrolyzing enzymes during host invasion (Cox et al., 2001; Ghannoum, 2000; Nespoulous et al., 1999). Also, phospholipase activity has been proposed to contribute to pathogenicity of bacterial and some fungal pathogens (Ghannoum, 2000; Leidich et al., 1998).

**Phospholipase A1 and A2**

Phospholipase A1 (PLA1) hydrolyzes the fatty acyl ester bond at the \( sn-1 \) position of the glycerol moiety of phospholipids, while phospholipase A2 (PLA2) removes the fatty acid at the \( sn-2 \) position. The action of PLA1 and PLA2 results in the accumulation
of free fatty acids and 2-acyl lysophospholipid or 1-acyl lysophospholipid, respectively. Cytosolic PLA2 associates with natural membranes in response to physiological increases in Ca\(^{2+}\) and selectively hydrolyses arachidonyl phospholipids. This enzymatic activity often initiates signal transduction and is regulated by the state of cell activation. In addition, PLA2 activity releases arachidonic acid from membranes in mammalian cells, which serves as a signaling molecule for cell-to-cell communication. Platelet-activating factor acetylhydrolase (PAF-AH), a subfamily of PLA2, are responsible for inactivation of platelet-activating factor through cleavage of an acetyl group (i.e., at the second position of glycerol in bioactive phospholipids) releasing lyso derivatives of phospholipid substrates and short fatty acids.

**Phospholipase B**

Phospholipase B (PLB; synonyms: lysophospholipase, lysophospholipase-transacylase) refers to an enzyme that can remove both \(sn-1\) and \(sn-2\) fatty acids. PLB has both hydrolase (fatty acid release) and lysophospholipase-transacylase (LPTA) activities. The hydrolase activity allows the enzyme to cleave fatty acids from both phospholipids (PLB activity) and lysophospholipids (Lyso-PL activity), while the transacylase activity allows the enzyme to produce phospholipid by transferring a free fatty acid to a lysophospholipid. PLBs contribute the virulence of the pathogenic yeast *C. albicans* and *C. neoformans* (Cox et al., 2001; Ghannoum, 2000; Leidich et al., 1998).

**Phospholipase C**

Phospholipase C (PLC) hydrolyzes the phosphodiester bond in the phospholipid backbone to yield 1,2-diacylglycerol and phospholipids depending on the specific phospholipid species involved. The products of PLC activities are involved in intracellular signaling processes or serve as sensors for the membrane status of a cell, thus affecting and regulating important cellular processes in eukaryotic cells (Van Leeuwen et al., 2004; Wera et al., 2001; Wang, 2004). The action of the phosphatidylinositol-specific PLC enzymes that catalyze the conversion of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) produces two well-characterized second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). It has
been suggested that plants and fungi have PLC signaling mechanisms that are different from animals, generating IP6 and phosphatidic acid (PA) as messengers instead of IP3 and DAG (Meijer and Munnik, 2003). Microbial infection in plants is known to result in the activation of plant PLC and the production of PA that serves as a signaling molecule.

**Phospholipase D**

Phospholipase D (PLD) enzymes catalyze the hydrolysis of phosphatidylcholine (PC) and are involved in membrane trafficking and cytoskeletal reorganization. PLD catalyzes hydrolysis of phospholipids to yield PA, which stimulates various cellular processes. As an example, the PLD encoded by the gene *PLD1/SPO14* in budding yeast is essential for meiosis, suggesting that formation of PA is involved in this form of cellular differentiation (Rose et al., 1995). Some studies suggest a role for this enzyme activity in intracellular membrane traffic. For example, Siddhanta A and Shields D (1998) showed that the accumulation of PA as a product of catalysis by PLD is a key process in the regulation of vesicle budding from the trans-Golgi compartment.

1.4.2.2. **Triglyceride lipases**

Triglyceride lipases (TGL) are lipolytic enzymes that hydrolyze ester linkages of triglycerides yielding fatty acids and glycerol. The lipase active site, the most conserved region in all these proteins is centered around a serine residue, which participates with a histidine residue and an aspartic acid residue in a charge relay system. These lipases are expressed and secreted during the infection cycle of some pathogens. In particular, *C. albicans* has a large number of TGLs (at least 10 genes), possibly reflecting broad lipolytic activity, which may contribute to the persistence and virulence of *C. albicans* in human tissue (Hube et al., 2000). The expression of the lipase genes was detected in media containing triglycerides as the sole carbon source and many of these genes were expressed during the yeast-to-hyphal transition (Fu et al., 1997; Hube et al., 2000). Other authors suggested that candidal species (*C. albicans*) considered to be more pathogenic had higher levels of lipase activity compared to less pathogenic ones (Ogawa et al., 1992). During early phases of pathogenic growth, the plant pathogen *Botrytis cinerea* produces a lipase that is essential for the infection of tomato leaves (Commenil et al.,
Another plant pathogen, *Alternaria brassicicola*, produces a spore surface-bound lipase while in contact with leaf surface waxes. The lipase activity is thought to contribute to spore adhesion and penetration of the plant surface (Berto et al., 1999). Recently, genetic studies have linked lipase production to virulence of *Fusarium graminearum* in wheat and maize (Voigt et al., 2005). Other lipolytic enzymes known as cutinases are postulated to aid in the degradation of cutin, a hydroxy fatty acid polyester that is a major structural component of the plant cuticle (Kolattukudy, 1970; also see section 1.3.1. Lipids and early stages of fungal development, above). In fact, cutinases are thought to be important in the invasion of plants by phytopathogenic fungi such as *Erisyphae graminis*, *Ascochyta rabiei* and others (Kolattukudy et al., 1995; Munoz and Bailey, 1998 and reviewed in Rogers et al., 1994). In addition, lipases may also allow pathogenic fungi to utilize host cell macromolecules as a source of nutrients (Salyers and Whitt, 1994).

### 1.4.2.3. Lipid modifying enzymes

Lipid modifying enzymes such as cyclooxygenases (COX) or lipoxygenases (LOX) play an important role in the formation of biologically active fatty acid derivatives (oxygenated signaling lipids or oxylipins). These lipids called eicosanoids are derived via the arachidonate cascade in animals. The eicosanoids include prostaglandins (PG), thromboxanes (TX) and leukotrienes. The arachidonic cascade is initiated by release of free arachidonate by activation of PLC and DAG lipase or PLA₂ followed by oxidation by cyclooxygenase or lipoxygenase. Oxidation of arachidonate to a hydroperoxide by LOX or COX is the first committed step in eicosanoid formation. Higher plants have a linolenate cascade that generally resembles the arachidonate cascade leading to formation of jasmonic acid, which can be converted into methyl jasmonate, known to be involved in plant defense responses to pathogen attacks.

Oxylipin production in fungi is ubiquitous and appears to play a role in fungal development and virulence. For example, the human pathogenic yeast *C. albicans* and *C. neoformans* are able to produce modified lipids called prostaglandins, which strongly enhance both cell viability and filamentation capacity (Deva et al., 2000, 2001; Noverr et al., 2001, 2003). The genes (*ppoA*, *ppoB* and *ppoC*) from *A. nidulans* encoding for
oxylipin-generating dioxygenases similar to mammalian cyclooxygenases are required for the production of oxylipins known as psi factors that influence spore development in this fungus (Hornsten et al., 1999; Huber et al., 2002; Tsitsigianis et al., 2004a, 2004b, 2005b). In addition, ssp1 from \textit{U. maydis} and \textit{Lds1} (linoleate diol synthase) from the wheat pathogen \textit{Gaumannomyces graminis} have also been proposed to have similar functions to mammalian cyclooxygenases. (for the effect of these proteins on fungal development see the section 4.2. Lipids in development and sporogenesis in fungi, above).

1.5. **Rationale and Aims of this Study**

At the start of this thesis work, relatively little was know about the \textit{U. maydis} – maize interaction in terms of the role of environmental signals or nutritional factors during infection. No signals, provided by the host plant or the environment, were known that would trigger the morphological transition between the budding non-infectious cell type and the filamentous infectious form. In addition, very little was known about the nutritional requirements of \textit{U. maydis} and other biotrophic fungi, and the contribution of host plants to this intriguing relationship.

1.5.1. **Hypotheses**

The hypotheses of this work are based on the initial discovery that lipids, particularly plant oils, induced filaments in \textit{U. maydis} that resembled the infectious cell type required to cause disease. The hypotheses state that: (I) fatty acids are signaling molecules that induce filamentous growth in \textit{U. maydis} with participation of the cAMP/PKA and Ras/MAPK cell signaling pathways known to regulate morphogenesis and pathogenicity in \textit{U. maydis}; and (II) the fatty acid-induced genes that influence the filamentous growth response may contribute to virulence of \textit{U. maydis}.
1.5.2. **Research objectives**

**Objective 1.** The first objective was to phenotypically characterize the lipid induction of filamentous growth in *U. maydis*. The specific goals were to examine the ability of different lipids and fatty acids to cause the dimorphic transition, to characterize the properties of the resulting filaments, and to determine whether components of the cAMP and MAPK signaling pathways were required (Chapter 2).

**Objective 2.** The second objective was to perform targeted gene disruption of candidate genes involved in fatty acid metabolism to investigate their roles in lipid-induced filamentation and host infection. These studies focused on genes encoding a peroxisomal multifunctional enzyme (*mfe2*), a mitochondrial 3-hydroxyacyl-CoA dehydrogenase (*had1*), and a phospholipase A2 (*lip2*) with the following goals:

**Peroxisomal multifunctional β-oxidation enzyme (*mfe2*)**

One major goal was to generate mutants that could not use fatty acids as a carbon source and to determine whether this type of mutant could still grow in plant tissue to cause disease. A gene for peroxisomal β-oxidation was selected for detailed analysis (Chapter 3).

**Mitochondrial 3-hydroxyacyl-CoA dehydrogenase (*had1*)**

A second goal was to test a candidate gene for a putative mitochondrial β-oxidation function for a role in filamentous growth and virulence (Chapter 4).

**Phospholipase A2 (*lip2*)**

Phospholipases are emerging as a potential class of novel virulence factors in pathogenic fungi and annotation of the genome sequence revealed several candidates. The *lip2* gene was selected because previous work in the laboratory suggested that expression of a PLA2 gene might be influenced by the cAMP pathway. (Chapter 5).
1.5.3. Significance

Generally, the significance of this research comes from the identification and characterization of a new chemical signal provided by fatty acids that triggers the morphological transition producing the infection filaments known to be essential for the pathogenicity of \textit{U. maydis}, and involves the conserved signaling pathways known to play a role in virulence of a number of fungi. The phenotypic analysis of mutant strains deleted in carefully selected genes expected to play a role in lipid/fatty acid metabolism provides a way to examine the role of lipid metabolism during morphological transitions and pathogenic development in \textit{U. maydis}. Overall, this research provides a framework to address the role of lipids in plant-fungal interactions and to understand the importance of lipid signaling and metabolism in fungal development and infection.
1.6. References


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2. LIPID-INDUCED FILAMENTOUS GROWTH IN USTILAGO MAYDIS

2.1. Introduction

Many plant pathogenic fungi are capable of altering their morphology to form infection-specific cell types to allow penetration and growth within plant tissues; this differentiation appears to be tightly controlled by signals from the host environment (Dean, 1997; Kronstad et al., 1998; Lee et al., 2003; Xu and Hamer, 1996). The identification of these signals and the pathways required for their recognition is an important challenge in understanding fungal pathogenesis. The basidiomycete fungus Ustilago maydis provides an experimental opportunity to investigate signaling in response to the host because this pathogen is obligately biotrophic only during a portion of its life cycle. U. maydis undergoes a dimorphic transition from a yeast-like, nonpathogenic cell type to a filamentous pathogenic form that is capable of colonizing maize tissue and inducing tumors. Infection of the host by U. maydis is an obligate part of the life cycle and entails several steps: (i) the exchange of pheromone signals between compatible haploid cells and the formation of conjugation tubes; (ii) fusion of cells to form a dikaryon that penetrates the plant surface; (iii) filamentous growth of the dikaryon in planta; (4) differentiation of the dikaryotic hyphae into diploid teliospores (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994). These spores are surrounded by an echinulated and melanized cell wall, and are produced in large numbers during infection (Banuett and Herskowitz, 1996; Christensen, 1963).

The obligate relationship between U. maydis and maize suggests that the fungus has evolved highly specialized mechanisms to recognize and adapt to the host environment. The regulation of developmental processes within the host is poorly understood, and host signals involved in the interaction are still unknown. Some features of infection hyphae observed in planta do not occur in vitro and these include extensive branching of hyphae, development of branch primordia resembling clamp connections.

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and random collapse of hyphal compartments compared to the hyphal collapse at sites distal to the growing tip in culture (Banuett and Herskowitz, 1996; Day and Anagnostakis, 1971; Snetselaar and Mims, 1994). In addition, prolonged growth of the filamentous dikaryon and teliospore development have not been observed in culture (Banuett and Herskowitz, 1996; Day and Anagnostakis, 1971). These observations suggest that the plant supplies crucial nutrients and/or signaling molecules that promote filamentation and sporogenesis. It has been proposed that the stage-specific expression of genes during development is achieved by repression of these genes during saprophytic growth and induction during biotrophic growth in the plant (Basse et al., 2000).

Evidence for in vitro progression of the fungus through the sexual cycle was obtained by growing U. maydis on embryonic maize cell cultures; although the components of the embryonic culture that promoted sexual development were not identified. (Ruiz-Herrera et al., 1999).

The identification of U. maydis mutants that are constitutively filamentous or defective for the filamentous phenotype led to the characterization of several genes encoding components of signaling pathways that influence pathogenicity (Barrett et al., 1993; Brachmann et al., 2001; Dürrenberger et al., 1998; Dürrenberger et al., 2001; Gold et al., 1994; Lee and Kronstad, 2002). Specifically, components of two signal transduction pathways were identified: the cyclic AMP/ protein kinase A (PKA) pathway and a Ras/mitogen-activated protein kinase (MAPK) pathway. Mutations in components of the cAMP signaling pathway influence sexual as well as pathogenic development. Strains deficient in either the Ga subunit of a heterotrimeric G protein (Gpa3; (Regenfelder et al., 1997), adenylyl cyclase (Uac1; Gold et al., 1994), or the catalytic subunit of PKA (Adr1; (Dürrenberger et al., 1998) are constitutively filamentous and are unable to cause disease symptoms. Mutants deficient in the regulatory subunit of PKA (Ubc1) display constitutive PKA activity, proliferate in plant tissue, but fail to produce tumors (Gold et al., 1997). Several components of the pheromone response (Ras/MAPK) pathway have also been identified including Ubc4 (MAPKKK), Fuz7 (MAPKK), Kpp2/Ubc3 (MAPK), and the pheromone response factor Prf1 (Andrews et al., 2000; Banuett and Herskowitz, 1994; Hartmann et al., 1996; Mayorga and Gold, 1999). Mutations in any of the MAPK pathway components result in faulty pheromone
signaling. Additional components of the pathways have been identified by suppressor screens; complementation of one suppressor mutation restored filamentous growth to a yeast-like \textit{adr1} suppressor mutant and identified the \textit{hgl1} gene (Dürrenberger \textit{et al.}, 2001). Hgl1 is thought to be a negative regulator of budding growth and an infectious dikaryon deficient for Hgl1 is defective in teliospore formation. Complementation of another suppressor mutation of the \textit{adr1} phenotype identified the \textit{ras2} gene, which encodes a member of the Ras family of small GTP-binding proteins (Lee and Kronstad, 2002). In \textit{U. maydis}, Ras2 regulates morphogenesis, pathogenesis and mating. Given the role of the cAMP and MAPK signaling networks in the regulation of filamentous growth, a hallmark aspect of \textit{U. maydis} biotrophy, they may be involved in sensing specific plant signals that trigger discrete stages of pathogenic development.

Characterization of signaling mutants with morphological defects revealed that growth with lipids (e.g., corn oil) as the sole carbon source resulted in a dimorphic transition in \textit{U. maydis}. In this report, I demonstrate that the signal transduction pathways known to regulate mating and virulence are involved in the response to lipids. Specifically, I found that mutants with defects in components of the cAMP/PKA pathway or the Ras/MAPK pathway failed to respond to triglycerides and fatty acids. In addition, I show that the ability to respond is suppressed by glucose and that \textit{U. maydis} exhibits an extracellular triglycerol lipase activity during growth on lipids. These results reveal a previously uncharacterized signal that influences the morphogenesis of \textit{U. maydis} and suggest that utilization of fatty acids as a carbon source is an important aspect of the ability of the fungus to undergo morphological changes. This discovery may have broad relevance for understanding metabolic aspects of the interactions between plants and fungal pathogens.

\section*{2.2. Results}

\subsection*{2.2.1. Growth on triglycerides promotes a filamentous morphology}

Wild-type haploid cells of \textit{U. maydis} generally exhibit yeast-like (budding) growth in standard culture medium that contains glucose as the carbon source (Holliday, 1974). In contrast, I found that haploid strains displayed a filamentous cellular
morphology in liquid medium supplemented with 1% corn oil as the sole source of carbon (Figure 2.1). Standard laboratory strains of two different mating types (521, albl and 518, a2b2) (Kronstad and Leong, 1989) both displayed the same response and started to produce hyphae after 12 hours of incubation. Hyphal formation was observed for approximately 50% of the cells in the population after 5 days of growth. A diploid strain (d132, ala2 blb2) was also grown in medium with corn oil and found to switch from budding to filamentous growth (Figure 2.1). Diploid strains of U. maydis that are heterozygous at the a and b mating-type loci are similar to the infectious dikaryon in their ability to cause disease symptoms, although diploids are pathogenic in the absence of mating. However, unlike dikaryons, diploid strains are capable of saprophytic growth in culture and resemble haploid strains in their budding growth on glucose-containing medium (Day and Anagnostakis, 1971; Holliday, 1974; Kronstad and Leong, 1989; Puhalla, 1968) (Figure 2.1).

Haploid wild-type strains were also grown in liquid medium supplemented with a pure triglyceride (trilinolein) as a sole carbon source. As expected, the strains produced filaments suggesting that the filamentous growth response to corn oil was due to the presence of triglycerides rather than minor contaminants in the oil (data not shown). In addition, other plant-derived triglycerides, including sunflower, canola and olive oil triggered the morphological change in both haploid and diploid cells (data not shown). Overall, our results indicate a novel influence of triglycerides on the dimorphic transition in U. maydis.

2.2.2. Fatty acids supplied as tweens promote filamentous growth

Corn oil is a mixture of triglycerides that contain various fatty acids esterified to glycerol. It is possible that the fatty acid components could be responsible for the filamentous growth response and that the fatty acids could display specificity because of differences in their saturation state and carbon chain length. As an initial examination of the role of fatty acids, I grew cells in liquid medium supplemented with one of four combinations of fatty acids supplied as soluble tweens.
Figure 2.1 Morphological response of *U. maydis* strains to triglycerides, fatty acids, and glucose.

The strains were examined after 5 days of growth in liquid MM with 1% glucose, 1% corn oil (mixed triglycerides), both 1% glucose and 1% corn oil, and 1% tween 40 (palmitate). Scale bar, 10μm.
The various combinations represented a selection of the fatty acids of different carbon chain length and saturation state that are typically found in plants (lauric 12:0, stearic 18:0, palmitic 16:0, oleic 18:1, linoleic 18:2 and linolenic 18:3 acid). *U. maydis* responded to all of these conditions by growing with a filamentous morphology indicating that no specificity was present at this level of analysis (Figure 2.1 and 2.2). To confirm that fatty acids are responsible for the morphological changes, both haploid and diploid wild-type cells were grown in medium supplemented with pure fatty acids (palmitic, oleic and linoleic). As expected, the production of filaments was observed in response to the fatty acids (Figure 2.2 and data not shown). However, filaments of the haploid strain (518) were short, and some resembled pseudohyphae (Figure 2.2), compared to the diploid strain (dl32) that produced a large network of long filaments. These results indicate that fatty acids promote the induction of filamentous growth. I also noted that provision of 1% glycerol as the carbon source triggered filamentous growth although the extent of growth was reduced compared to the medium with tweens (not shown). Therefore, *U. maydis* may respond to each component of triglycerides and other non-carbohydrate carbon sources may influence morphogenesis. The production of filaments in glycerol may reflect a general response to less preferred carbon sources.

The wild-type cells grew well in lipids added to a concentration of 1% (corn oil or tweens) although higher cell densities were obtained for cultures in media with tweens (data not shown). This was possibly due to better availability because of the higher solubility of tweens compared with the oil. To investigate the possible metabolic and signaling influences of lipids on filamentous growth, I grew haploid and diploid wild-type cells in liquid medium supplemented with a range of concentrations of tween 40. Hyphal formation was observed in cultures containing concentrations as low as 0.01% (approximately 800 nM palmitic acid) (Figure 2.3). However, the cells did not grow well in lower concentrations of tween 40, and this may have impaired their ability to respond (Figure 2.3). To examine the response at lower tween concentrations in a situation where growth could occur, I added arabinose (a non-repressing five-carbon sugar) to the medium with each of the concentrations of tween 40.
Figure 2.2  Cellular morphology of *U. maydis* wild-type strains in the presence of fatty acids.

Haploid and diploid wild-type strains were grown for 5 day in liquid MM supplemented with (A) 1% tween solutions containing saturated (palmitate and laurate) or unsaturated (oleate+linoleate and oleate+linolenate) fatty acids, and (B) 1% pure fatty acid (linoleic acid). Scale bar, 10μm.
**Figure 2.3** Morphological response of *U. maydis* to various concentrations of fatty acids.

Haploid wild-type strain was grown in liquid MM supplemented with various concentrations of tween 40 (palmitate) either alone or with 0.5% arabinose to allow sufficient growth. 

(A) Morphological response at four representative concentrations of tween 40 shown to demonstrate the range of responses. Cells in medium supplemented with arabinose only were grown for a control. Scale bar, 10 μm. (B) Growth measurements at OD600. The mean values and standard deviations (bars) of the measurements from three independent experiments are given.
Strikingly, the production of filaments was observed in the arabinose cultures grown in much lower concentrations of tween 40 compared with cultures grown in the same concentrations without arabinose (Figure 2.3). Specifically, filaments were observed at concentrations down to 0.00005%, representing approximately 4 nM of palmitic acid in the medium. Altogether, these results suggest that fatty acids provide a carbon source and a chemical signal that promotes filament formation.

2.2.3. **Invasive filamentous growth occurs on solid medium supplemented with fatty acids**

I also investigated the growth of haploid and diploid strains on agar medium supplemented with palmitic acid as the carbon source (added as tween 40). Within 48 hrs, the cells had started to invade the agar by producing distinct invasive filaments (Figure 2.4). Branching of the invasive filaments inside the agar was clearly evident and in this regard resembled infection hyphae *in planta* (data not shown). Invasive filaments were not observed for strains on agar medium supplemented with glucose as the carbon source after washing the cells off the surface (Figure 2.4). It is possible that the invasive growth was triggered in response to the fatty acids or the hydrophobicity of the solid medium. I also noticed that crystals formed inside the fatty acid-containing agar, but not the glucose medium (Figure 2.4). These crystals may result from metabolism of the fatty acids or diffusible compounds released in the presence of fatty acids. *U. maydis* is known to secrete glycolipids termed ustilagic acids when grown in oils (Boothroyd B, 1956; Fluharty and O'Brien, 1969; Lemieux RU, 1951; Spoeckner S, 1999). Ustilagic acid forms crystals similar to those seen on the fatty acid-containing agar plates. Overall, these observations demonstrate the ability of *U. maydis* to produce invasive hyphae in the presence of fatty acids.
**Figure 2.4 Invasive hyphal growth of* U. maydis* induced by fatty acids.**
Haploid wild-type cells were grown on solid medium supplemented with 1% tween 40 (palmitate) or 1% glucose for 5 days. Cellular morphology on glucose- and fatty acid-containing medium is shown before washing the cells off the surface (top panel). After washing the plate, cells that have invaded the medium are visible only on the fatty acid-containing plate (bottom panel).
2.2.4. Components of two signaling pathways are required for the response to lipids

The cAMP/PKA pathway and a MAPK signaling pathway are known to influence filamentous growth and virulence in *U. maydis* (Kronstad et al., 1998); (Dürrenberger et al., 1998) (Lee and Kronstad, 2002); (Spellig et al., 1994). I therefore tested mutants with defects in these pathways for their ability to respond to triglycerides (corn oil) and fatty acids (palmitate as tween 40) (Figure 2.1) as well as pure triglyceride (trilinolein) (data not shown). Mutants with low PKA activity, such as those with defects in adenylyl cyclase (*uacl*) or the catalytic subunit of cAMP-dependent PKA (*adrl*), grow with a constitutively filamentous phenotype. Growth with lipids in the culture medium did not affect the filamentous phenotype of these mutants (data not shown). Previous work correlated low PKA activity with filamentous growth (Dürrenberger et al., 1998; Gold et al., 1994). Consistent with these observations, the *ubcl* mutant defective in the regulatory subunit of cAMP-dependent PKA (with high PKA activity) did not produce filaments in response to either triglycerides or fatty acids (Figure 2.1) suggesting that the unregulated PKA activity in this mutant prevented the morphological response. A mutant with a defect in the *hgl1* gene also did not form filaments on the lipid medium (Figure 2.1). The *hgl1* gene is thought to encode a regulatory protein downstream of PKA that functions to negatively regulate budding (Dürrenberger et al., 2001). All of the strains were also tested for the response to other fatty acids supplemented as tween 20, 80 or 85, and they responded in the same manner as observed in the medium with palmitate (tween 40) (data not shown).

Mutants with defects in the components of the Ras/MAPK pathway were also tested for their ability to respond to lipids. This pathway is interconnected with the cAMP pathway and plays a role in the filamentous growth response to mating pheromone (e.g., formation of conjugation tubes) as well as filamentous growth in planta (Andrews et al., 2000; Krüger et al., 1998; Mayorga and Gold, 1998, 1999). I found that mutations in *ras2*, *fuz7* (encoding a MAPKK) and *ubc3* (encoding a MAPK) all blocked the filamentous growth response to lipids and that only the mutant defective in the pheromone response transcription factor (encoded by *prfl* gene) formed filaments in the
presence of corn oil and palmitate (Figure 2.1). Therefore, the Prf1 protein that is required for the transcription of genes involved in mating was not required for the filamentous growth response to lipids. The same responses to fatty acids found in the medium with palmitate (tween 40) were also observed in media supplemented with other tweens (tween 20, 80 and 85) as a sole carbon source (data not shown). Our results indicate that the signal(s) triggering filament production in response to lipids may be transduced by Ras2 via a MAP kinase cascade that includes Fuz7 and Ubc3. Epistasis experiments indicate that Ras2 is upstream of Fuz7 and Ubc3 (Lee and Kronstad, 2002). Overall, our findings establish a connection between the filamentation response to lipids and the U. maydis cAMP and Ras/MAPK signaling networks that are known to function in pheromone response, filamentous growth in host tissue, and virulence.

2.2.5. Glucose suppression of the filamentous growth response

Most microorganisms can utilize a variety of carbon sources, but glucose is often preferred and metabolized first (Carlson, 1999; Ullmann, 1996). Because glucose repression is well known in fungi, I was interested in determining whether the presence of glucose would interfere with triglyceride-induced filamentous growth. The response to corn oil in liquid medium was compared to the response to glucose alone, and to corn oil and glucose together for wild-type strains and strains defective in components of the cAMP/PKA and Ras/MAPK pathways. All of the strains exhibited the budding phenotype when grown in glucose medium (Figure 2.1). Suppression of filamentous growth was observed in the liquid medium with both corn oil and glucose for all of the haploid and diploid wild-type strains, and for the signaling pathway mutants that responded to lipids (Figure 2.1). In addition, the growth rate of the wild-type cells in corn oil medium was similar to that in glucose medium as a sole carbon source (data not shown). I also studied the effects of other sugars (sucrose, fructose and L-(+)-arabinose) on the filamentous growth response. The same strains that were used for the glucose suppression study were grown in liquid medium supplemented with corn oil with sucrose, fructose or arabinose. Sucrose suppressed the formation of filaments, exhibiting the same effect on the response as glucose. The cells grown with corn oil in the presence of fructose or arabinose responded by switching from budding to filamentous growth (data
not shown and Figure 2.3, respectively). However, partial suppression of the filamentous growth response was observed in the cultures with fructose. These results reinforce the idea that carbon source can have a major influence on morphogenesis in *U. maydis*.

2.2.6. The morphology of lipid-induced filaments resembles *in vivo* filaments

The morphology of the fatty acid-induced filaments was examined more closely to determine whether they share features with the dikaryotic or diploid filaments observed *in planta* during the infection process. The morphological features of the latter two cell types are indistinguishable during growth in the host (Banuett and Herskowitz, 1996). For comparison, I grew the diploid strain d132 in liquid medium supplemented with palmitic acid (Tween 40). The fatty acid-induced filaments produced *in vitro* were long and showed both extensive branching and septum formation (Figure 2.5A and B), features that are typical for filaments observed *in planta* (see Figure 2 of Banuett and Herskowitz, 1996 and Figures 5 and 6 of Snetselaar and Mims, 1994). The hyphal branches arose at irregular intervals and at various angles to the main filaments. Cross walls were clearly visible and were observed separating the branches from the main hyphae and the individual cylindrical cells within the hyphae (Figure 2.5A and B). These features were confirmed by calcofluor staining (Figure 2.5B).

Hyphal collapse is also a typical feature of growth *in planta*. For the *in vitro* filaments, the hyphal collapse occurred randomly in different parts of the hyphae to generate both thick (filled with cytoplasm) and thread-like (lacking cytoplasm) segments (Figure 2.5A and B). Hyphal collapse is also known to occur for dikaryotic cells in culture; however, in this situation, generally only hyphal tips contain cytoplasm but the rest of the hyphal cells are empty (Banuett and Herskowitz, 1996; Day and Anagnostakis, 1971; Snetselaar and Mims, 1992). Branch primordia with an associated Y-shaped septum that separates the primordium from the main hypha were also observed for the *in vitro* grown filaments (Figure 2.5B). These structures are similar to the clamp connections of other basidiomycetes that participate in maintaining the dikaryotic stage (Banuett and Herskowitz, 1996), and therefore were previously named as clamp-like structures.
Figure 2.5 *In vitro* filaments of *U. maydis* induced by fatty acids.

Diploid wild-type cells were grown in liquid MM supplemented with 1% tween 40 (palmitate) as a sole carbon source for 5 days. (A) DIC images illustrating the morphologies of the *in vitro* filaments revealed the presence of the hyphal features typical for *in planta* filaments (white arrows). The photographs are representative images of filaments from 6 independent experiments. The scale bar, 10μm. (B) Staining with calcofluor white to analyze septum formation within the filaments confirmed the branching, and presence of septa and clam-like structures. Scale bar, 5 μm. For comparison collapsed hyphae (ch) and hyphae with cytoplasm (h) are shown.
Given that all of the characteristics described above are typical for the filaments formed in the plant during infection, and the hyphal branching and clamp-like structures form only \textit{in planta} (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994), our results suggest that the fatty acids in the culture medium may reflect one of the nutritional conditions that the fungus encounters in the plant. These conditions may contribute to fungal ramification by promoting the development of highly branched filaments, although the host environment is of course more complex and other factors such as contact with host cells may have an important influence on the fungus (Podila et al., 1993).

\textbf{2.2.7. Triglycerol lipase activity is found in lipid-grown cultures}

Lipases are thought to play a role in virulence during fungal infections by assisting in cell penetration, tissue colonization, and by allowing fungal catabolism of host lipids (Berto \textit{et al.}, 1999; Commenil \textit{et al.}, 1995; Gottlich \textit{et al.}, 1995; Weber \textit{et al.}, 1999). I anticipated that \textit{U. maydis} would secrete extracellular lipase during growth on corn oil and that the lipase activity might be regulated by the signaling pathways that control filamentous growth. To examine these possibilities, the culture supernatants of various strains grown in triglyceride or tween-containing media were examined for extracellular lipolytic activity. As expected, lipase activity was detected in culture supernatants obtained from the haploid and diploid wild-type strains and the fatty acid-responsive mutant strains (e.g., prfI mutant) grown in liquid minimal medium with tween 40 (palmitate) (Figure 2.6). Interestingly, the mutant strains that did not respond to lipids (including the ubc1, hgl1, ras2, fus7 and ubc3 mutants) also exhibited lipase activity (Figure 2.6), but the activity was lower than in wild-type strains and the fatty acid-responding mutants. The non-responding strains were still able to grow in the presence of fatty acids, possibly due to a low level of lipolytic activity that enables them to use the fatty acids as a carbon source. The \textit{ubcl} mutant that is defective in the regulatory subunit of PKA grew poorly, did not form filaments on lipid-containing medium (corn oil and tween 40) and exhibited very low levels of lipase activity in the culture supernatants (Figure 2.6).
Figure 2.6 The extracellular lipase activity in cultures of *U. maydis* during the response to fatty acids.

The graph represents specific enzyme activity in culture supernatants of haploid and diploid wild-type strains, and mutants defective in the cAMP and MAPK signaling pathway grown in medium with tween 40 (palmitate) as the sole carbon source. The control sample does not contain a source of extracellular lipase (negative control). The enzyme activity of lipase from *C. rugosa* (Sigma) was used as a positive control. The bar charts show the mean and standard deviation from three independent experiments. Note that *ubcl* mutant grows poorly in lipid medium. For example, in a representative experiment the *ubcl* mutant recorded on OD600 of 0.7 compared to the wild-type strain value of 2.7.
These results suggest that cAMP signaling may be involved in the ability to use oils as a carbon source and that the gene(s) encoding the lipase activity may be regulated by PKA. In general, these results suggested that secreted lipases may contribute to the response of the fungus to triglycerides and fatty acids, and may ultimately contribute to virulence.

2.3. Discussion

The ability to switch between budding and filamentous growth is well documented in several fungal pathogens of plants and animals including *U. maydis*, *Ophiostoma (Ceratocystis) ulmi*, *Histoplasma capsulata*, *Blastomyces dermatitidis*, and *Candida albicans* (Gold et al., 1994; Kronstad and Staben, 1997; Lengeler et al., 2000; Madhani et al., 1997; Maresca and Kobayashi, 2000; Medoff, 1987; Wang and Heitman, 1999). Morphogenesis is known to be associated with virulence in some of these fungi (e.g. *C. albicans*; Lo et al., 1997) and in some cases the environmental signals that influence dimorphism have been identified (e.g., N-acetylglucosamine (Singh et al., 2001), serum (Feng et al., 1999) and farnesoic acid (Oh et al., 2001) for *C. albicans*). Our observation that triglycerides or fatty acids promote the dimorphic transition in *U. maydis* is intriguing because the filamentous cell type is the biotrophic, obligately parasitic phase of the life cycle. This phase coincides with the sexual development of the fungus because it is initiated by mating and results in the formation of the diploid teliospores that are capable of meiosis. The hypothesis that arises from our results is that the response to lipids could be an important component of the infection process and that the morphological adaptation to lipids as a carbon source may reflect one of the nutritional conditions that the fungus encounters in the plant. This hypothesis is supported by our observations that the signaling components known to control virulence are also required for the lipid response and that the *in vitro* and *in vivo* filaments share morphological similarities.

2.3.1. Lipid-induced filaments resembled the cells found in plant tissue.

I found that the filaments produced in response to lipids displayed characteristics typical of the filaments that develop during infection; in particular, I observed branching
and clamp-like structures that were previously thought to occur only *in planta* (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1994). The branching is thought to contribute to the extensive hyphal proliferation observed within the tumor tissue in advance of teliospore formation and branching has been proposed as an indicator of the response to a plant signal (Banuett and Herskowitz, 1996). Other features of the infectious filaments such as branched hyphae with visible crosswalls, and collapsed hyphal sections were also observed on solid medium with lipids. The cells on solid medium also demonstrated the ability to invade the substrate in response to the presence of lipids and this may be relevant to the ability of the filaments to invade host tissue during infection.

### 2.3.2. Role of the nutrient-sensing cAMP pathway in morphogenesis

The cAMP pathway in other fungi and in mammals plays a key role in nutrient sensing and metabolism. For example, the cAMP pathway is a major glucose-signaling pathway in *Saccharomyces cerevisiae*, and plays a central role in the control of metabolism and proliferation (Rolland *et al.*, 2001). Also, filamentation induced by a nitrogen limitation in *S. cerevisiae* is controlled by the cAMP pathway (Gimeno *et al.*, 1992; Gimeno and Fink, 1994; Lorenz *et al.*, 2000). In our study, a connection between the response to lipids and cAMP signaling came from the observation that the *ubcl* mutant (defective in the regulatory subunit of PKA) grew poorly in lipids (corn oil and tween 40) and did not respond morphologically. The mutant also did not exhibit lipase activity in the culture supernatants from growth medium with Tween 40. These results suggest that cAMP signaling is involved in the ability to use oils as a carbon source. It is possible that the *ubcl* mutant may not produce lipases or may not be able to activate lipases as a result of high unregulated PKA activity in the mutant. This finding suggests that the lipase gene(s) lies downstream of the PKA, although other explanations are possible such as an indirect influence of PKA on fatty acid metabolism, a direct influence on other metabolic enzymes or other metabolic defects. A role for the cAMP pathway is further indicated by the finding that a downstream target of PKA, the *hgl1* gene product (Dürrenberger *et al.*, 2001), was also required for the morphological response and showed reduced lipase activity compared with wild-type cells.
One model for the behavior of *U. maydis* is that growth on a lipid carbon source results in reduced levels of cAMP, low PKA activity and filamentous growth. Certainly, a correlation between low PKA activity and filamentous growth have been well established (Dürrenberger *et al.*, 1998; Gold and Kronstad, 1994). Interestingly, *ubcl* mutants fail to respond to lipids, but they are filamentous as dikaryons in the plant, although the cells do not proliferate as extensively as the wild-type dikaryon and do not induce tumors. Perhaps these observations indicate a separation of perception of a signal indicating the presence of a host, as determined by the MAPK pathway, and evaluation of the nutritional status as perceived by the cAMP pathway (Gold *et al.*, 1997). In this scenario, the early stages of filamentous growth such as mating and invasion might be controlled by the MAPK pathway; later stages such as branching and ramification in host tissue could be regulated by the cAMP pathway to allow fungal proliferation prior to tumor formation. Additionally, the utilization of lipid carbon sources might allow the fungus to proliferate in host tumor cells after carbohydrates have been exhausted (in preparation for sporulation).

Unfortunately, little is known about lipid metabolism and carbon source utilization by *U. maydis* during the biotrophic stage of its life cycle. In this context, the cAMP-regulated *sspl* gene was recently found to be abundantly expressed in mature teliospores of *U. maydis*; this gene encodes a protein with similarity to linoleate diol synthase (LDS) and prostaglandin G/H synthases (cyclooxygenases) from mammals (Huber *et al.*, 2002). It is thought that Ssp1 plays a role in the mobilization of storage lipids to ensure teliospore maturation. A connection between cAMP signaling and the degradation of lipid reserves during appressorium formation has also been demonstrated for the rice blast fungus *Magnaporthe grisea* (Thines *et al.*, 2000).

2.3.3. **Glucose suppression suggests a metabolic component for lipid-induced filamentation**

I found that glucose and other sugars such as sucrose suppressed the switch from budding to filamentous growth in response to triglycerides and fatty acids. This result was not surprising because glucose is known to repress the transcription of many genes in fungi. For example, the *FOX1* gene, which encodes an acyl-CoA oxidase involved in the
β-oxidation of fatty acids, is repressed by glucose in *S. cerevisiae* (Stanway et al., 1995). Also, low glucose-medium induces increased activity for the *SNF1* (sucrose-non-fermenting) protein kinase in *S. cerevisiae*. Snf1 is responsible for the derepression of many glucose-repressed genes and the kinase is known to inactivate the key enzyme (acetyl-CoA carboxylase) involved in fatty acid biosynthesis (Woods et al., 1994). This glucose-regulated repression of genes involved in β-oxidation of fatty acids and fatty acid biosynthesis may contribute to an efficient utilization of glucose. The presence of glucose also inhibits the expression of the *LIP1* gene that encodes a lipase in *C. albicans* (Fu et al., 1997). For *U. maydis*, the presence of lipids and the absence of glucose were required for filamentous growth. If the lipid response is relevant to growth in planta, then the timing of utilization of different carbon sources may be important and may contribute to development of specialized nutritional interfaces between the invading hyphae and the host cells (Hahn and Mendgen, 2001). Bhaskaran et al., (1991) found that host extracts contain a glycoprotein (presumably from the plant cell wall) that triggers filamentous growth in another smut fungus, *Sporisorium reilianum*. Interestingly, glucose also inhibited this response.

### 2.3.4. Could a lipid signal act through the Ras/MAPK pathway?

Both the cAMP/PKA and the Ras/MAP kinase pathway are known to regulate pathogenesis in *U. maydis*. However, the roles of the cAMP-dependent and MAP kinase pathways appear to be antagonistic with respect to filamentous growth (Krüger et al., 1998). The activated Ras/MAP kinase pathway stimulates production of filaments (Lee and Kronstad, 2002), but high PKA activity is associated with budding growth (Dürrenberger et al., 1998; Gold et al., 1994). Given the role for cAMP and PKA in the response to nutrients, it may be the case that the Ras/MAPK pathway functions to perceive lipids as signals. A similar interconnection of the two pathways occurs in *S. cerevisiae* to control pseudohyphal growth (Mosch et al., 1999; Rupp et al., 1999; Lorenz and Heitman, 1997; D'Souza and Heitman, 2001; Lengeler et al., 2000).

To examine the possibility that lipids may represent a signal as well as a carbon source for *U. maydis*, I tested the response of the fungus to decreasing levels of tween 40
in the presence and absence of arabinose as a supplementary carbon source. I found that a low level of tween (approximately 4 nM) prompted a response, suggesting that lipids may represent signaling ligands as well as a carbon source. I speculate that the lipid signal may be transduced at least in part via the components of the Ras/MAPK pathway because I found that mutants lacking the ras2, fuz7, and ubc3 genes did not respond to triglycerides or fatty acids. The transcription factor Prf1 proved to be not essential for the morphological response to lipids suggesting that a different transcription factor(s) is involved.

2.3.5. Lipid signaling in fungi

A role for lipids in morphogenesis has been described in several fungi. For example, plant surface lipids (waxes) have been reported to provide signals for plant-fungus interactions and are thought to induce pathogenic development in fungi including Colletotrichum species (Podila et al., 1993; Macko, 1981). Kolattukudy et al., (1995) reported that plant surface lipids contain both inducers and inhibitors that influence spore germination and appressorium formation required for infection. In addition, several studies indicate that modified fatty acids or related lipids are important for sexual and asexual development in filamentous fungi (Nukina and . 1981; Goodrich-Tanrikulu et al., 1998). For example, linoleic acid and its derivatives were found to play a role in spore formation for several fungal species (Calvo et al., 1999; Hyeon, 1976; Katayama, 1978; Rai, 1967). Fatty acid signaling molecules called psi factors (hydroxylated linoleic acid) influence growth, spore formation and aflatoxin production in Aspergillus nidulans (Champe et al., 1987; Mazur, 1991). Recently, oleic acid-derived psi factors were also found to affect the asexual to sexual spore ratio in A. nidulans (Calvo et al., 2001). Plant-derived fatty acids (hydroperoxylinoleic acids) also contribute to spore development in Aspergillus spp. (Calvo et al., 1999). Finally, the human pathogenic fungi C. albicans and Cryptococcus neoformans produce prostaglandins (oxygenated unsaturated cyclic fatty acids) by conversion of exogenously supplied as well as endogenously produced arachidonic acid; the prostaglandins enhance both cell viability and filamentation capacity for C. albicans (Noverr et al., 2001). These studies highlight the additional
possibility that derivatives of fatty acids, perhaps generated by fungal activities, could serve as signaling molecules.

2.3.6. Summary

In summary, we found that *U. maydis* forms filaments in culture in response to lipids. We believe that this finding may be relevant to infection of the plant because the components of two signaling pathways required for pathogenesis are also needed for the response to lipids. Furthermore, the morphological features of the filaments formed *in vitro* resemble those of the infectious dikaryon observed *in planta*. We believe that lipid metabolism is important for the response because we observed suppression in the presence of glucose. In addition, the low level of lipids required for the response suggests that they are acting as ligands to trigger the morphological change. These novel observations may have general implications for understanding both the regulation of fungal dimorphism and the pathogenesis of biotrophic fungi.

2.4. Material and Methods

2.4.1. Strains and growth conditions

*U. maydis* strains used in this study are listed in Table 2.1. The strains were grown at 30°C on potato dextrose agar or broth (Difco), or in liquid minimal medium (MM) (Holliday, 1974) supplemented with lipids. These included 1% vegetable oil (corn, olive, canola or sunflower), or 1% trilinolein (Sigma) as triglyceride sources, and 1% tween (polyethylene sorbitans of fatty acids) 20, 40, 80 or 85 (Sigma) or 1% linoleic acid, oleic or palmitic acid (Sigma), as fatty acid sources. The tweens have the following compositions: tween 20 (containing mainly lauric, myristic and palmitic acid), tween 40 (palmitic, stearic and oleic acid), tween 80 (oleic, linoleic, and palmitic acid) and tween 85 (oleic, linolenic and palmitic acid). Other carbon sources added to liquid MM included 1% glucose, 1% sucrose, 1% fructose or 0.5% and 1% arabinose (Sigma). Filament production was assayed in the following tween 40 concentrations: 1% (representing approximately 80 μM of palmitic acid in the medium), 0.5% (40 μM),
0.25% (20 μM), 0.1% (8 μM), 0.05% (4μM), 0.025% (2 μM), 0.01% (800 nM), 0.005%
(400 nM), 0.001% (80 nM), 0.0005% (40 nM), 0.0001% (8 nM), and 0.00005% (4 nM).
The same range was also used in the presence of 0.5% arabinose. In the standard assay
for filamentous growth, 5 mL of liquid MM was inoculated with 10⁶ cells and incubated
for five days at 30°C with shaking. Cell growth in liquid MM was monitored by
measuring absorbance at OD600.

2.4.2. Invasion assay

To characterize filamentous growth on solid medium, 10⁵ cells were plated on
MM containing 1.5% agar and supplemented with 1% tween 40 or 1% glucose. The
plates were photographed and then screened for invasive filamentous cells by washing
the plate surface with running water as described (Palecek P. Sean, 2001) after 1, 3, and 5
days of growth at 30°C. The remaining cells that had invaded the agar were
photographed.

2.4.3. Microscopy, staining, and photography

Cells were examined with a Zeiss Axioplan 2 Fluorescent microscope. Bright
field, differential interference microscopy (DIC), and fluorescent images were captured
with a DVC camera and processed electronically with Northern Eclipse imaging
software. A Nikon dissecting microscope was used at 10X magnification to record
invasive filamentous growth on solid MM supplemented with tween 40. Photographs
were obtained with a Nikon Coolpix 990 digital camera and processed with Adobe
Photoshop 7. To visualize septa, filaments from the liquid medium were incubated in a
10-ml preparation of calcofluor white (Fluorescent brightener 28; Sigma, 20 μg/ml) for 15
min.

2.4.4. Triglycerol lipase assay

The activity of extracellular triglycerol lipase in cultures grown in MM
supplemented with 1% tween 40 was determined by a turbidimetric enzyme assay (von
The supernatant obtained by centrifugation of cultures at 13,200 rpm for 15 min was used as a source of the extracellular lipase activity. A standard reaction contained 300 μL of supernatant added to 2% (v/v) Tween 20 in 20 mM Tris-HCl (3.6 mL), pH 8.0, and 120 mM CaCl$_2$ (0.1 mL). The mixture was incubated for 30 min at 37°C. The lipase activity is expressed in units per mg protein of a culture supernatant. Protein concentrations were determined using a protein assay kit (BioRad). Purified lipase from *Candida rugosa* (Sigma) was employed as a positive control.

2.5. Tables

**Table 2.1 Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>518 (001)</td>
<td>$a_2 b_2$</td>
<td>(Kronstad and Leong, 1989)</td>
</tr>
<tr>
<td>521 (002)</td>
<td>$a_1 b_1$</td>
<td>(Kronstad and Leong, 1989)</td>
</tr>
<tr>
<td>33</td>
<td>$a_1 b_1 Δuac1$</td>
<td>(Gold et al., 1994)</td>
</tr>
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<td></td>
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<td>(Dürrenberger et al., 1998)</td>
</tr>
<tr>
<td>111</td>
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<td>(Gold et al., 1994)</td>
</tr>
<tr>
<td>3011</td>
<td>$a_1 b_1 Δhgl1 hyg^+$</td>
<td>(Dürrenberger et al., 2001)</td>
</tr>
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<td>6</td>
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<td>(Lee and Kronstad, 2002)</td>
</tr>
<tr>
<td>FB1-26</td>
<td>$a_1 b_1 Δfuz7 hyg^+$</td>
<td>(Banuett and Herskowitz, 1994)</td>
</tr>
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<td>(Mayorga and Gold, 1999)</td>
</tr>
<tr>
<td>UM0407</td>
<td>$a_1 b_1 Δprf1 phleo^+$</td>
<td>(Kohno, De Maria, and Lee, unpublished data)</td>
</tr>
<tr>
<td>d132</td>
<td>$a_1/a_2 b_1/b_2$</td>
<td>(Kronstad and Leong, 1989)</td>
</tr>
</tbody>
</table>
2.6. References


3. THE MULTIFUNCTIONAL β-OXIDATION ENZYME IS REQUIRED FOR FULL SYMPTOM DEVELOPMENT BY THE BIOTROPHIC MAIZE PATHOGEN *USTILAGO MAYDIS*\(^2\)

3.1. Introduction

*Ustilago maydis* causes a common smut disease on maize (*Zea mays*) that can result in an economically significant reduction in yield (Pataky, 1991; Pataky, 1990). Sexual development of the fungus is tightly interconnected with infection, and involves several morphological transitions (Banuett and Herskowitz, 1996). The key transition for initial infection and for subsequent colonization of the plant is the production of the filamentous dikaryon. This cell type is established upon recognition between compatible haploid cells by pheromone exchange leading to the formation of conjugation tubes known as mating filaments that initially grow on the plant surface. Subsequently, these cells fuse to form a dikaryotic filament, which is capable of invading host tissue. The mating filaments and the dikaryotic filaments prior to penetration are straight filaments that do not branch. Once in the plant, the fungus proliferates extensively to produce a large network of branched filaments. Mating and filamentation are controlled by conserved cAMP/protein kinase A (PKA) and mitogen activated protein kinase (MAPK) signaling cascades; these control distinct stages of the disease process by largely unknown mechanisms (Andrews et al., 2000; Banuett and Herskowitz, 1994; Barrett et al., 1993; Brachmann et al., 2001; Durrenberger et al., 2001; Durrenberger et al., 1998; Gold et al., 1994; Gold et al., 1997; Hartmann et al., 1996; Lee and Kronstad, 2002; Mayorga and Gold, 1999). Recently, we showed that lipids and fatty acids induce filamentation in *U. maydis* (Klose et al., 2004). This response may be relevant to infection because the components of the PKA and MAPK signaling networks are required for both the dimorphic transition and the response to lipids. Additionally, the

\(^2\) A version of this chapter has been accepted for publication. Klose, J., and J. W. Kronstad. 2006. The Multifunctional beta-oxidation Enzyme Is Required for Full Symptom Development by the Biotrophic Maize Pathogen *Ustilago maydis*. Eukaryot Cell 22, 22. (on-line early)
morphological features of the lipid-induced filaments formed in vitro resembled those of the infectious dikaryon observed in planta.

_U. maydis_ is an obligate biotrophic pathogen during the sexual phase of its life cycle. Infectious filaments initially invade epidermal cells and grow intracellularly surrounded by the intact host cell plasma membrane (Snetselaar and Mims, 1994; Snetselaar and Mims, 1992). At this stage, early disease symptoms such as chlorosis and anthocyanin pigmentation are visible on infected maize plants. Later in development, filaments grow mostly intercellularly around cells of the vascular bundle (Snetselaar and Mims, 1994). Following penetration and proliferation, the fungus induces tumors in which the cells exhibit extensive branching, hyphal fragmentation and the formation of melanized teliospores (i.e., sexual spores). The fungal cells in tumor tissue are embedded in thin-walled parenchymatous plant cells, which have been shown to lack plastids (Callow and Ling, 1973). To date, little is known about fungal genes that control or are required for development in the plant, or about host signals that may contribute to pathogen development. It is clear that the biotrophic fungal life style requires an intimate relationship with the plant because the host cells remain alive while metabolites are redirected to feed the pathogen. In this regard, _U. maydis_ establishes long lasting interactions with maize, often without causing any visible damage to invaded cells and without provoking a defense response (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1993). Therefore it must have strategies to overcome resistance, either by masking its intrusion, suppressing host defense, and/or inducing specific host genes for the establishment of biotrophy. It has been shown, that drastic changes in transcript levels of maize genes related to metabolism and development occur during _U. maydis_ infection (Basse, 2005). In general, it seems likely that sensing the nutritional state of the host environment during biotrophic growth is critical for disease development by _U. maydis_.

Our previous work indicated that lipids act as signals and as carbon sources to promote filamentous growth in culture for _U. maydis_ (Klose et al., 2004). Given the relationship between filamentous growth and pathogenesis for _U. maydis_, and the abundance of lipids in plant tissue, it is possible that lipids are also important signals and/or carbon sources during maize infection. I have shown that _U. maydis_ secretes
lipase activity in culture to break down lipids (Klose et al., 2004), and assuming that this activity is expressed during infection, the released fatty acids could be further degraded via β-oxidation, a process by which fatty acids are broken down to acetyl-CoA by sequential removal of two carbon units in each oxidation cycle. A relationship between peroxisomal metabolic function and phytopathogenesis has been previously tested in the hemibiotrophic fungus *Colletotrichum lagenarium* (Kimura et al., 2001). In this fungus, disruption of a gene for peroxisome biogenesis resulted in a defect in appressorium-mediated plant infection but the mutant retained the ability for invasive growth *in planta*. In addition, analysis of the transcriptome of the obligate biotrophic fungus *Blumeria graminis* at different stages in the life cycle revealed coordinate regulation of enzymes involved in primary metabolism, including lipid degradation enzymes (Both et al., 2005). However, in this case, the fungus appears to use lipids stored in conidia to fuel colonization of host tissue via appressorium formation, and storage lipids are regenerated during growth in the host. These studies leave open the question of whether β-oxidation is required for successful infection by obligate fungal biotrophs. β-oxidation could also contribute to the production of modified fatty acids that are known to influence development in fungi. For example, oleic acid and linoleic acid, and their derivatives, influence growth and spore formation in filamentous fungi (Calvo et al., 2001; Champe et al., 1987).

In this study, I made use of the fact that *U. maydis* is obligately biotrophic during the sexual stage of its life cycle but can also be cultured in the laboratory as a saprophyte. These properties allowed us to compare the contribution of peroxisomal β-oxidation to fungal morphogenesis and growth in culture with the requirement for this process during biotrophic infection. Specifically, I constructed and characterized *U. maydis* mutants lacking the *mfe2* gene that encodes the multifunctional enzyme for the second and third steps in peroxisomal β-oxidation. I found that the *mfe2* gene was required for the switch to filamentous growth on some but not all fatty acids, and that it was needed for growth on long chain fatty acids in culture. Inoculations of seedlings and developing ears with the mutants resulted in fewer tumors and delayed sporulation, indicating that *mfe2* was necessary for full symptom development. Overall, these results suggest that lipids represent an important but not essential carbon source during biotrophic growth and raise
the possibility that lipid utilization by *U. maydis* may influence additional aspects of infection such as signal perception or host defense.

3.2. Results

3.2.1. Identification of genes encoding peroxisomal β-oxidation enzymes

As an initial step to characterize the role of β-oxidation in *U. maydis* pathogenesis, the sequences of the enzymes involved in the process in *S. cerevisiae* were used to identify homologs in the *U. maydis* genomic sequence, recently completed at the Broad Institute (with annotation at the Munich Information Center for Protein Sequences, MIPS). The first committed step in peroxisomal β-oxidation is catalyzed by acyl-CoA oxidase (ACOX), a unique marker enzyme for non-mitochondrial β-oxidation in eukaryotic cells (Kunau et al., 1995). There are five *U. maydis* genes (Um04324, Um02208, Um01966, Um02028, and Um04833) predicted to encode acyl CoA oxidases (homologs of the yeast protein Fox1). The second and third steps in β-oxidation are catalyzed by a multifunctional enzyme that is encoded by FOX2 (CAA82079) in *S. cerevisiae* (Hiltunen et al., 1992). *U. maydis* has one homolog encoded by a gene designated mfe2 (Um10038; multifunctional enzyme type 2). The Mfe2 protein shows 47% identity and 63% similarity to the yeast Fox2 protein over 721 amino acids. The fourth enzymatic reaction is catalyzed by 3-ketoacyl CoA thiolase and there are three homologs (Um03571, Um01090 and Um02715) with similarity to the yeast protein Fox3. Because a single gene (*mfe2*) was found to encode the second and third steps, this gene was chosen for subsequent deletion to generate mutants that would be unable to utilize fatty acids as a carbon source. Such mutants would allow an investigation of whether lipid metabolism had an influence on fatty acid-induced filamentation and virulence in *U. maydis*.

The *mfe2* gene encoded a predicted polypeptide of 911 amino acids with a duplicated region for the two dehydrogenase domains in the first half of the protein (PF00106/IPR002198; amino acid regions 19 to 249 and 326 to 499) (Figure 3.1). A separate hydratase domain (PF01575/IPR002539) was present in the C-terminal region. It is possible that the dehydrogenase domains may contribute to different substrate
specificities; for example, the first domain is most active with long- and medium-chain substrates, and the second domain with short chain substrates in *S. cerevisiae* (Qin et al., 1999). The C-terminal domain encoding the 2-enoyl-CoA hydratase 2 is common to all multifunctional enzymes of the MFE 2-type (Figure 3.1). The mammalian peroxisomal Mfe2 enzyme contains only one dehydrogenase domain with broad substrate specificity (both long- and short-chain substrates) (Novikov et al., 1994) (Figure 3.1A). At least three types of peroxisomal targeting sequences (PTSs) direct proteins to peroxisomes in yeast including PTS1 and PTS2, and a third type that has not been well characterized (de Hoop and Ab, 1992; Purdue and Lazarow, 1994). For example, some of the acyl-CoA oxidases in *C. albicans* or *S. cerevisiae* do not possess PTS1 or PTS2 and may be targeted to peroxisomes by either of two internal, redundant sequences (Kamiryo et al., 1989; Small et al., 1988). Neither PTS1 nor PTS2 were found in the *U. maydis* Mfe2 sequence and the enzyme may possess the third type of PTS. In addition to the high amino acid sequence identity of Mfe2 to the yeast Fox2 protein, BLAST analysis confirmed sequence similarity to other characterized peroxisomal multifunctional enzymes from the following fungi: *Candida tropicalis* (P22414, 47% identity) (Moreno de la Garza et al., 1985), *Neurospora crassa* (CAA56355, 54% identity) (Fossa et al., 1995), *Yarrowia lipolytica* (AAF82684, 55% identity) (Smith et al., 2000), and *Glomus mosseae* (Q9UVH9, 53% identity) (Requena et al., 1999) (Figure 3.1B).

### 3.2.2. Induction of *mfe2* gene expression by fatty acids

Fatty acids induce expression of the genes/enzymes involved in β-oxidation and transcription of the corresponding genes is subject to glucose repression in yeast (Veenhuis et al., 1987, Stanway et al., 1995, Kunau and Hartig, 1992, Luo et al., 1996). I established that the *mfe2* gene fits this pattern because RNA blot analysis revealed that transcript levels were relatively high upon growth in medium with oleic acid (C18:1) or linoleic acid (C18:2) as the sole carbon source (Figure 3.2). Transcript levels were lower in cells grown on these fatty acids in the presence of glucose, and in cells grown on glucose as the sole carbon source. These results indicate that *mfe2* transcription is influenced by carbon source as expected for a β-oxidation function.
Figure 3.1 Organization of the peroxisomal multifunctional enzymes (type 2).
(A) Organization of the catalytic domains of *U. maydis*, *S. cerevisiae* and human peroxisomal Mfe2 enzymes. (B) Sequence alignment of the conserved catalytic domains from the *U. maydis* Mfe2 protein with fungal, (*C. tropicalis* (CANTR, P22414), *S. cerevisiae* (SACCE, CAA82079), *N. crassa* (NEUCR, CAA56355) and *Y. lipolytica* (YARLI, AAF82684)), and human (P51659) homologs. (HD) = (3R)-hydroxyacyl-CoA dehydrogenase domain, (H2) = 2-enoyl-CoA hydratase 2 domain.
Figure 3.2 RNA blot analysis of mfe2 transcript levels in the presence of fatty acids.

(A) Total RNA was isolated from the wild-type (a2b2) cells grown in the minimal medium containing either glucose, fatty acid, or fatty acid and glucose. Total RNA was also isolated from the Δmfe2 a2b2 cells grown in the minimal medium containing fatty acids. The RNA blot was hybridized with a probe for the mfe2 gene. (B) The RNA blot stained with 0.04% methylene blue to show total RNA loading.
3.2.3. Targeted deletion of the mfe2 gene

To investigate the role of β-oxidation during the growth of U. maydis in culture and in planta, I performed a targeted gene deletion by replacing the entire open reading frame of mfe2 with a 3.8 kb gene cassette conferring resistance to the antibiotic hygromycin B. The deletion vector was introduced into two U. maydis wild-type strains of opposite mating type (albl and a2b2) to allow subsequent analysis of mating and virulence. Deletion of the gene was confirmed by colony PCR and Southern blot analysis (not shown) and two independent mutants in each strain were used for subsequent experiments. RNA blot analysis revealed the absence of the mfe2 transcript in the mutants thereby confirming complete gene inactivation (Figure 3.2).

3.2.4. Growth and Filamentation on Long Chain Fatty Acids (LCFA)

I initially characterized the ability of the mfe2 mutants to undergo a morphological transition from budding to filamentous growth in the presence of lipids and fatty acids. Palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acids were chosen for this study because our previous analysis demonstrated that wild-type strains respond to these fatty acids (Klose et al., 2004), and these are also the most abundant fatty acids present in maize plants (Watson, 2003; Watson, 1987). As expected, the wild-type strains grew as filaments in the presence of all of the LCFA tested and in the presence of corn oil (Figure 3.3A). The mutant strains responded to corn oil, palmitic acid and oleic acid by switching from budding to filamentous growth thus indicating that the mfe2 gene was not generally required for fatty acid-induced filamentation (Figure 3.3A). This observation suggests that it is possible to separate the utilization of fatty acids as a carbon source from their function as signaling molecules in the filamentous response. However, the mutants did not respond morphologically to linoleic acid thus raising the possibility that specific fatty acids may contribute to the production of signals that influence filamentous growth.
Figure 3.3 Morphology and growth of mfe2 mutant strains on fatty acids differing in carbon chain length and saturation state.
(A) Cellular morphology of the wild-type (a2b2) and mutant (Δmfe2 a2b2) strains in response to glucose, lipids (corn oil), LCFA (palmitic, oleic, and linoleic), VLCFA (erucic, arachidic and arachidonic), SCFA (caproic) and MCFA (lauric and myristic). The cells were visualized by differential interference contrast optics (DIC, left) and by epifluorescence after staining cell walls with calcofluor (right). (B) The ability of the wild-type and mutant strains to grow on fatty acid-containing agar medium. The cells were spotted in decreasing concentration from $10^6$ to $10^2$. All the agar plates contain tergitol to facilitate the solubility of the fatty acids. (C) Total number of the wild-type (a2b2, black bars) and mutant (Δmfe2 a2b2, white bars) cells in culture supplemented with glucose, corn oil, and fatty acids as a sole carbon source. The bars represent the average number of cells from 3 independent experiments.

In addition to the general filamentation response, differences in the morphologies of the wild-type cells were observed in the presence of different LCFA (Figure 3.3A). Wild-type cells grown in oleic acid developed large rounded central cells with long branched filaments emerging from the center. Interestingly, the oleic acid-grown cultures of the wild-type cells were also pigmented with a dark brown to black color (data not shown). Wild-type cells grown on linoleic acid developed an extensive network of robust and branched filaments with many rounded cells. Some of these cells had lobed ends and resembled the filaments observed in the early stages of sporulation in plant tissue (Banuett and Herskowitz, 1996). In palmitic acid, the wild-type cells grew as long straight hyphae that resembled the straight mating filaments and the infectious dikaryotic filaments that form before and after the fusion of compatible strains, respectively. Furthermore, these hyphae produced very few branches, a phenotype observed only on palmitic acid. Overall, these results support the idea that long chain, unsaturated fatty acids may be important in promoting branching in wild-type cells and thus may contribute to extensive proliferation during growth in plant tissue. Our findings in culture suggest that linoleic acid may be particularly important in this role.

The mfe2 mutants were also tested for their ability to utilize fatty acids by growing the cells on fatty acid-containing agar medium supplemented with LCFA (palmitic, oleic and linoleic) and in a liquid medium supplemented with triglycerols (corn
oil) and LCFA (palmitic, oleic and linoleic acids) as the sole carbon source (Figure 3.3B and C). The mutants were able to grow on corn oil (Figure 3.3C). This was not surprising because the triglycerols consist of fatty acids esterified to a glycerol backbone, and *U. maydis* is known to be able to use glycerol as a carbon source (Klose et al., 2004). The wild-type strains grew well on corn oil, oleic acid and linoleic acid when compared to glucose (Figure 3.3C). The mutant strains were unable to efficiently utilize oleic and linoleic acids as a carbon source, although a low level of residual growth was observed for both of these fatty acids when grown in liquid medium (Figure 3.3C). The mutants grew poorly on palmitic acid, although the growth of the wild-type strain was also reduced suggesting that *U. maydis* generally does not efficiently utilize this fatty acid. In addition, the mutant strains failed to grow on all of the LCFA tested when spotted in a range of cell concentrations on fatty acid-containing agar plates (Figure 3.3B). In contrast, the wild-type strain grew well on corn oil, oleic and linoleic acid when compared to growth on glucose (Figure 3.3C). Overall, I conclude that loss of *mfe2* results in growth defects on LCFA as expected for strains with a defect in β-oxidation.

### 3.2.5. Growth and Filamentation on Very Long Chain Fatty Acids (VLCFA)

Fungi require peroxisomal β-oxidation to break down VLCFA and I therefore investigated the ability of these fatty acids to induce filamentation in *mfe2* mutants. The wild-type and mutant strains were inoculated into medium with one of the following fatty acids as a sole carbon source: arachidic (C20:0), arachidonic (C20:4), and erucic (C22:1). All of these VLCFA induced filamentous growth in the wild-type strains, although the filaments in arachidonic acid had a slightly different appearance with primarily short branching cells (Figure 3.3A). In contrast, none of the VLCFA induced filamentation in the *mfe2* mutants. These mutants failed to grow on VLCFA, and the wild-type strain also showed poor growth on arachidic and arachidonic acids, but not erucic acid (Figure 3.3C). In addition, jasmonic acid was also tested for the ability to induce filamentation, however it completely abolished the growth of both the wild-type and mutant cells (data not shown). I also noted that both the wild-type and mutant strains produced extracellular needle-like crystal structures in the medium with erucic acid (data not
shown). These crystals could be glycolipids, because *U. maydis* is known to produce extracellular glycolipids called ustilipids (Boothroyd et al., 1956; Hewald et al., 2005; Lemieux et al., 1951) that are visible as needle-like precipitates. Interestingly, similar long needle-like crystals were also observed when wild-type strains were grown in the medium supplemented with both oleic and linoleic acids, but not in the medium containing either one of these fatty acids alone (data not shown). Taken together, these observations suggest that the fungus may need peroxisomal β-oxidation to break down VLCFA to produce shorter fatty acyl chains or to contribute to the production of putative modified fatty acids that might promote filamentous growth.

### 3.2.6. Growth and Filamentation on Short Chain Fatty Acids (SCFA) and Medium Chain Fatty Acids (MCFA)

The ability of SCFA, caproic acid (C6:0) or MCFA, lauric (C12:0) and myristic (C14:0) acids to induce filamentation was also examined (Figure 3.3A). The wild-type strain responded by growing as short distorted branched cells in all of the SCFA and in lauric acid, but produced long branched filaments in myristic acid. Intriguingly, the *mfe2* mutants did not display a filamentous response to any of these fatty acids, except myristic acid. I also explored the ability of the *mfe2* mutants to grow on SCFA and MCFA and found that these carbon sources supported only a limited growth for both the wild-type and mutant strains (Figure 3.3C). SCFA and MCFA have previously been shown to inhibit growth in some fungi (Maggio-Hall and Keller, 2004). Growth was not completely abolished but was limited to approximately four cell doublings. These results suggest the existence of another utilization pathway (e.g., mitochondrial β-oxidation) or residual growth on stored lipids.

### 3.2.7. Cellular Lipid Accumulation Is Influenced by Loss of Peroxisomal β-oxidation

The loss of Mfe2 function clearly alters the growth and filamentation response of *U. maydis* to different fatty acids. A defect in the homologous gene, *FOX2*, in *S. cerevisiae* mutants also alters lipid body production. I therefore used Nile red to observe
and compare the accumulation of lipid bodies in the wild-type and mutant cells grown on glucose, as well as on inducing (myristic, oleic) or non-inducing (linoleic) fatty acids. The yeast-like cells grown on glucose produced four to six large lipid bodies in the wild-type cells, and two to four large and some small lipid bodies in the mutant cells (Figure 3.4A). Overall, the accumulation pattern of the lipid bodies appeared to be unaffected in the mfe2 mutants grown on glucose. The lipid bodies varied in size and number in the fatty acid-induced filamentous cells depending on the fatty acid present in the growth medium. Specifically, the lipid bodies were very small and numerous in both wild-type and mutant cells on myristic acid (Figure 3.4A). This result suggests that both cell types accumulate and store myristic acid in lipid bodies that have structural differences compared with those formed in glucose-grown cells. The oleic acid-induced filaments of wild-type cells contained large lipid bodies in the central cells, and only diffuse fluorescence in the filaments branching from these cells (Figure 3.4A). In contrast, the lipid bodies in the mutant cells on oleic acid were dispersed and visible as small intensely stained droplets throughout the short branched cells (Figure 3.4A). A more striking difference was observed for the cells grown on linoleic acid. In this case, the wild-type filaments were filled with numerous large and small lipid bodies throughout their entire length, but the mutant cells did not produce lipid bodies and did not change their morphology (Figure 3.4A). Only a diffuse fluorescence was observed within the yeast-like cells of the mutant. (Figure 3.4A).

The ability of cells to respond to fatty acids (e.g., oleic acid) by forming filaments in comparison to the yeast-like cells found on glucose is particularly interesting and may be relevant to the filamentous growth observed in planta. I therefore performed a more detailed comparison of the lipid accumulation in the different morphological types by electron microscopy and by chemical analysis. I examined the mfe2 mutants by TEM to compare the size and numbers of the lipid bodies with those in wild-type strains (Figure 3.4B). I found that very few lipid bodies were observed in the yeast-like wild-type cells grown on glucose, while the mfe2 mutant cells appeared to produce more and larger lipid bodies in this medium (Figure 3.4B). In contrast, the lipid bodies in the oleic acid-induced wild-type filaments were more easily identified and were often found in clusters (Figure 3.4B).
Figure 3.4 Intracellular lipids in *U. maydis*.

(A) Cellular lipid accumulation in *U. maydis* wild-type and *mfe2* mutant strains grown on glucose and various fatty acids. The wild-type and *mfe2* mutant strains were grown in minimal medium supplemented with either glucose, myristic (C14:0), oleic (C18:1), or linoleic acid (C18:2) as a sole carbon source. The internal lipids accumulated in lipid bodies were stained using the lipidspecific fluorescent dye Nile red, and visualized using epifluorescence. The fungal cells produced large (arrowhead) to small (arrow) lipid bodies that varied in number depending on carbon source. Scale bar = 10 μm. (B) An abundance of lipid bodies produced in the *mfe2* mutant strain grown on oleic acid as a sole carbon source. TEM observation of lipid bodies in wild-type cells (*a2b2*) and *mfe2* mutant (*Δmfe2 a2b2*) cells grown on glucose and oleic acid. Cells were grown on glucose medium overnight and then transferred into oleic acid medium. After 18 hr, the oleic acid-grown cells were fixed and processed for TEM. Scale bar = 500 nm. (L) = Lipid body.
There was a striking difference in the accumulation of the lipid bodies in the \textit{mfe2} mutant strain grown on oleic acid compared to the wild-type strain (Figure 3.4B). The numerous lipid bodies almost completely filled the mutant cells. Therefore, these cells appeared to accumulate but not metabolize the exogenous fatty acids from the medium as expected for cells defective in \(\beta\)-oxidation.

Total internal lipids were also extracted from wild-type and \textit{mfe2} mutant cells grown either on glucose or oleic acid to examine whether the \textit{mfe2} deletion alters intracellular lipid composition and to evaluate potential changes in fatty acid composition during fatty acid-induced filamentation. The extracted internal lipids were converted to methyl esters, and analyzed using gas chromatography to determine fatty acid species in the different cell types. This analysis revealed differences in total abundance of fatty acids between the wild-type and the \textit{mfe2} mutant cellular lipids (Table 3.1). Specifically, more linoleic (C18:2) than oleic (C18:1) acid was found in wild-type and mutant yeast-like cells grown on glucose. Oleic acid was the predominant fatty acid in filamentous cells from oleic acid medium, accounting for 79\% of total fatty acids in the wild-type and 90\% in the \textit{mfe2} mutant cells. Strikingly, linoleic acid comprised only 9\% of total fatty acids in the oleic acid-grown mutant cells. The mutant accumulated oleic acid in higher levels relative to linoleic acid than did the wild-type strain. The ratio between oleic and linoleic acid in the mutant cells was 10:1 compared to 4:1 in the wild-type cells. This finding correlates with the structural data showing an excessive accumulation of lipid bodies in \textit{mfe2} mutants grown on oleic acid (Figure 3.4B). One possibility is that the mutant is unable to breakdown oleic acid available in the environment, but it is still able to accumulate the fatty acid to excessive amounts compared to the wild-type cells. The yeast-like cells grown on glucose contained more linoleic acid compared to the filamentous cells grown on oleic acid, which contained higher levels of endogenous oleic acid. Moreover, both glucose-grown wild-type and mutant strains, which exhibit yeast-like morphology, accumulated more saturated palmitic acid (C16:0) compared to oleic acid-grown cells, accounting for 23\% of total lipids in the wild-type cells and 17\% in the mutant cells. Palmitic acid was found only in trace amounts in the oleic acid-induced filamentous cells. Taken together, these comparisons indicate that the \textit{mfe2} mutants are different from wild-type cells in terms of their accumulation of lipid bodies and their fatty
acid composition when grown on oleic acid. These differences and the growth defects of the mutant on fatty acids raised the possibility that the mutants would have reduced growth during infection. I addressed this possibility in inoculation experiments with both vegetative tissue (seedlings) and floral tissue (developing ears), as described in the following sections.

3.2.8. Mfe2 Is Not Required for the Production of Mating Filaments

*U. maydis* must mate to produce the filaments required to infect plant tissue. The mating filaments are easily visualized on charcoal-containing agar plates where they generate white, fuzzy colonies in contrast to the smooth colonies produced by haploid yeast-like strains (Banuett and Herskowitz, 1994). The mfe2 mutants produced white, aerial hyphae during mating thus indicating a positive mating reaction (Figure 3.5A). The mating filaments were scraped from the surface of the plate and examined microscopically. The mating filaments produced from the mixture of compatible mfe2 mutant strains did not exhibit any differences in cellular morphology with respect to the wild-type strains (Figure 3.5B). The filamentous morphology resembled that of wild type in that they were straight with collapsed hyphal compartments. The cells at the growing tips were elongated with visible chitin accumulation (Figure 3.5B). Overall, mating is unaffected in mfe2 mutants suggesting that β-oxidation does not play an essential role in the formation of the infectious cell type.

3.2.9. Loss of mfe2 Results in Attenuation of Virulence during Plant Infection

The inoculation of corn seedlings with compatible wild-type and mfe2 mutant strains revealed that the mutants were clearly attenuated for virulence, although mild disease symptoms were observed (Table 3.2). The mutants were still able to induce tumors on leaves and basal parts of the plant stems, and to produce teliospores. However, only 27% of the plants infected with the mixture of compatible mfe2 mutant strains developed tumors, compared to 88% of the plants infected with the compatible wild-type strains (Table 3.2).
Figure 3.5 Mating filaments produced by compatible \textit{mfe2} mutant strains during mating.

(A) Mating test of wild-type (\textit{a1b1} and \textit{a2b2}) and \textit{mfe2} mutant strains. Each strain was spotted on charcoal-containing medium, and the compatible strains were mixed in the center of the plate to assess the mating reaction. The positive mating reaction is represented by the production of white filaments that are visible on the dark medium. (B) Dikaryotic filament formed by a cross of the wild-type and \textit{mfe2} mutant strains on charcoal-containing medium after 48 hrs. Both wild-type and mutant strains produced unbranched mating and dikaryotic filament with collapsed sections of hyphal cells. The images were captured using DIC optics (left panel) or epifluorescence (right panel) to visualize calcofluor-stained cell walls. Scale bar = 10 \mu m.
A large proportion (73%) of the plants infected with the mutant strains developed only chlorosis and anthocyanin pigmentation. I also tried to remediate the virulence defect by including 1.5% or 3% glucose with the inoculum as a way to potentially bypass nutritional defects in the mutant strains. This treatment did not change the outcome in terms of the severity of disease symptoms (data not shown). However, it is unclear whether the glucose would persist for a sufficient time and in the proper location to support all stages of fungal growth during infection. The teliospores produced in tumors of the plants infected with the \textit{mfe2} mutants were also tested for their ability to germinate and no obvious difference was found compared to spores from the wild type inoculations (data not shown). These data suggest that \(\beta\)-oxidation is not essential for \textit{U. maydis} to complete the life cycle \textit{in planta}, but it is required for robust growth of the fungus in the host and the development of full disease symptoms.

\subsection*{3.2.10. \textit{mfe2} mutant strains do not proliferate extensively \textit{in planta}}

Given the virulence defect in \textit{mfe2} mutants, I investigated the importance of the \textit{mfe2} gene during the initial penetration and subsequent proliferation of the infectious filaments \textit{in planta}. Epidermal peels from maize leaves infected with the compatible wild-type and \textit{mfe2} mutant strains were collected at one, four and seven days post-inoculation, and stained with calcofluor for visualization of fungal cells. Straight mating filaments and the infectious dikaryotic filaments that result from compatible mating reactions were observed on the surface of the maize leaves of both wild type and mutant infections one day after inoculation. Many of the wild-type, dikaryotic filaments had entered the plant epidermis, mostly through stomata. In contrast, the dikaryotic filaments of the mutant had not yet penetrated the epidermal layer at this stage. After four days post inoculation, many of the mutant filaments had penetrated the plant tissue and had started to grow inside the plant tissue; however, no obvious branching of the filaments was visible. At this time, the wild-type filaments showed extensive growth within the plant cells and many branched hyphal growing tips were observed (Figure 3.6A). After seven days post inoculation, a large network of the mutant filaments was observed within the plant cells. However, no obvious branching of the invading filaments was observed (Figure 3.6C).
Figure 3.6 Hyphal morphology in planta.

(A) Wild-type filaments growing within plant tissue 7 days after inoculation with compatible wild-type strains (albl X a2b2). The filaments branched (arrowheads), and could penetrate epidermal cells through a stoma (arrow). The tip of the penetrating hypha is out of the focal plane. (B) Yeast-like cells of compatible mfe2 mutant strains on the epidermal surface of a maize leaf. Some of the cells were elongated and started to produce conjugation tubes (ct) possibly in response to a mating partner. The cells often clustered around stomata, and once a dikaryotic filament was formed, it sometimes penetrated through the stomata (arrow). (C) mfe2 filament growing within plant tissue. The mutant filaments were often observed on the epidermal surface or within plant tissue without branching, exhibiting typical straight mating-like hyphal morphology. Epidermal peels from maize leaves were examined using DIC (top panel) and epifluorescence (bottom panel) to visualize the calcofluor-stained cells. Scale bar = 10 μm.
Many yeast-like cells of the mutant strains were still found on the epidermal surface, often clustered around stomata (Figure 3.6B). In contrast, the wild-type infection resulted in large networks of filamentous cells that were extensively branched. These results suggest that deletion of \textit{mfe2} reduced the ability of the fungus to produce the highly branched filaments that are crucial for fungal proliferation in host tissue.

3.2.11. Deletion of \textit{mfe2} Delays Teliospore Development within Tumor Tissue

\textit{U. maydis} infects any above ground part of the plant and symptoms are particularly dramatic in developing ears of mature plants. To examine the virulence defect seen in young seedlings more closely, I infected developing ears of two to three month old plants with compatible wild-type and mutant strains. The plants infected with the mutants showed a delay in symptom formation that was consistent with the defect seen in infected seedlings (Figure 3.7). The individual kernels of cobs infected with wild-type strains had developed into large black tumors containing an abundance of mature, melanized teliospores by 14 days after inoculation (a total of 13 cobs with tumors were collected in three independent experiments) (Figure 3.7A, B and C). In contrast, the cobs infected with the mutant strains were white and cross sections of these tumors indicated that they contained only immature spores (a total of 12 cobs with tumors were collected in three independent experiments) (Figure 3.7A, B and C). Specifically, the fungus within the tumors was found in several of the stages of development that occur prior to maturation of teliospores (Banuett and Herskowitz, 1996). These stages included bloated hyphae with lobed hyphal tips embedded within a mucilaginous matrix (appearing approximately 7-8 days postinoculation in wild-type cross), fragmentation of sporogenic hyphae producing individual fragments containing cells in the process of rounding (appearing approximately 9 days postinoculation in wild-type cross), cells with different morphologies that are in the process of forming teliospores and immature teliospores which are not yet melanized (appearing approximately 12 days postinoculation in wild-type cross) (Figure 3.7C).
Figure 3.7 Teliospore production of mfe2 mutants is compromised in mature tumors.

(A) Tumors collected from infected mature maize plants 14 days postinoculation. The cross of compatible wild-type strains resulted in production of mature tumors in 14-day period in contrast to the immature tumors produced by the cross of mfe2 mutant strains. (B) Subset of tumors collected from mature maize plants 14 days postinoculation. All mfe2 mutant tumors were white in appearance and contained sporogenic hyphae indicating that fungal development was not yet complete. (C) The cross section of the tumors shown in (A). The wild type “black” tumors were filled with melanized teliospores. The mutant “white” tumors were filled mostly with sporogenic hyphae and immature sexual spores that were not yet melanized, and therefore have not completed development. Scale bar = 10 µm.
The development of teliospores by the mutant strains were also examined at 20 days after inoculation to assess whether spores would eventually form at wild-type levels. A high proportion of melanized teliospores were observed within the 20-day old tumor tissue indicating that the mutants were delayed in their development compared to the wild-type strains. These observations support the conclusion that the loss of \textit{mfe2} influences the pathogenic development of \textit{U. maydis}.

\subsection*{3.3. Discussion}

I have shown in this report that deletion of the \textit{mfe2} gene encoding a multifunctional \(\beta\)-oxidation enzyme influences both the morphological response to fatty acids and the growth of \textit{U. maydis} on these substrates. Furthermore, loss of \textit{mfe2} altered lipid accumulation and fatty acid composition within mutant cells. These observations on cells grown in culture established a foundation to examine growth of the mutant in the host and I observed attenuated virulence in both maize seedlings and developing ears. The simplest explanation is that the defect in virulence results from a metabolic deficiency that prevents proper utilization of host nutrients and therefore delays extensive proliferation \textit{in planta}. However, it is also possible that specific fatty acids present in the host play a signaling role that is important for successful infection-related development (e.g., filamentation) for \textit{U. maydis}. A defect in lipid metabolism in the pathogen could interfere with the processing of these putative signals. Additionally, fungal lipid metabolism might influence plant lipid signaling and indirectly interfere with or promote a defense response that alters filamentous proliferation. As discussed below, these results have implications for understanding fungal biotrophy with respect to nutritional requirements, pathogen perception of the host environment and plant defense.

\subsection*{3.3.1. Fungal Phytopathogenesis and Lipid Utilization}

Evidence from several phytopathogenic fungi indicates that lipid metabolism is critical during the early stages of infection that involve spore germination, production of infection structures (i.e., appressoria formation), and penetration. For example, lipid droplets move to the appressorium following germination of conidia in \textit{M. grisea}, and
degradation in the vacuole appears to contribute to the glycerol accumulation required for generating turgor pressure during penetration (Thines et al., 2000). A similar mobilization and utilization of lipid reserves may occur in *Colletotrichum* species (Barbosa et al., 2006). For the biotrophic fungus *Blumeria graminis*, microarray data also indicate that genes for lipid catabolism are highly expressed during early stages of infection and decrease in expression in later stages (Both et al., 2005). Consistently, storage lipids in conidia of *B. graminis* are used during penetration and colonization of the host and reaccumulate later in the life cycle when new conidia are formed. Transcriptome analyses also indicate that lipid catabolism is important throughout the germination and penetration stages of infection of *B. graminis* (Thomas et al., 2002; Thomas et al., 2001). In a genetic test of the role of peroxisomal function in fungal phytopathogenesis, Kimura et al., (2001) showed that loss of peroxisomal function through mutation of the *clapex6* gene resulted in a defect in appressorial penetration by *C. lagenarium*. However, the fungus was still able to proliferate in the host when introduced through wounds, suggesting that lipid catabolism was not required for invasive growth. This pathogen displays biotrophy early in infection with a subsequent switch to necrotrophic growth that may overcome the requirement for peroxisomal function. Although the importance of β-oxidation in phytopathogenesis has not been studied in detail, accumulating evidence supports the importance of the glyoxylate cycle that produces glucose from the acetyl-CoA that results from the breakdown of fatty acids. Specifically, the glyoxylate cycle is required for full virulence in the phytopathogenic fungi *Tapesia yallundae*, *Leptosphaeria maculans*, *M. grisea* and *Stagonospora nodorum*, and the human fungal pathogen *C. albicans* (Bowyer et al., 2000; Idnurm and Howlett, 2002; Lorenz et al., 2004; Lorenz and Fink, 2001; Solomon et al., 2004; Wang et al., 2003). Furthermore, enzymes for lipid degradation, including secreted lipases, also contribute to virulence in some phytopathogens such as *Botrytis cinerea*, *Alternaria brassicicola* and *Fusarium graminearum* (Berto et al., 1999; Commenil et al., 1995; Voigt et al., 2005).

In contrast to the situation in other phytopathogens, peroxisomal β-oxidation appears to be less important during the early stages of disease development in *U. maydis*. Specifically, the processes of teliospore germination, haploid cell mating to form the
infectious cell type, and plant surface penetration, were unaffected in the \textit{mfe2} mutants. \textit{U. maydis} also differs from many other well-studied phytopathogenic fungi in that it does not produce true appressoria (i.e., rounded, melanized structures), but instead produces appressorium-like swellings at the tips of infectious dikaryons to penetrate the plant surface. \textit{U. maydis} also does not make obvious haustoria to acquire nutrients from the host (Snetselaar and Mims, 1993; Snetselaar and Mims, 1994). In contrast, our analysis indicated that loss of \textit{mfe2} influenced later stages of disease development, including extensive proliferation of branched hyphae and sporulation \textit{in planta}. \textit{U. maydis} generally infects actively growing meristematic tissue in maize, resulting in tumors that are often found in the immature, expanding tissue at the base of the leaf (Callow and Ling, 1973; Wenzler and Meins, 1987). Glycolipids and phospholipids would potentially be available to the fungus in this tissue and these generally contain the following fatty acids: C16:0, C18:0, C18:1, C18:2, and C18:3 (Hawke et al., 1974; Leech et al., 1973). Traces of C14:0, C16:1 and C20:0 are also found. Fatty acids are synthesized in plastids in developing plant tissue, and the amount of lipid increases in parallel with plastid development in green developing maize leaves (Leech et al., 1973; Moore and Troyer, 1983). A similar fatty acid composition is also found in maize kernels (Hsing et al., 1993; Ratcliff et al., 1993). It is not yet clear whether \textit{U. maydis} would have access to these lipids during infection and tumor formation. Upon infection, the fungus initially grows intracellularly in epidermal cells, parenchyma cells and cells of the vascular bundles. Later, the fungus grows mostly intercellularly as highly branched hyphae, some of which protrude into plant cells (Snetselaar and Mims, 1994). Banuett and Herskowitz (1996) also described the proliferation of hyphae within host cells with the eventual rupture of the cells during sporulation. In general, examination of tumor sections reveals aggregates of fungal hyphae in the process of sporulation surrounded by hypertrophied host cells that appear mainly empty (Snetselaar and Mims, 1994). Callow and Ling (1973) also reported that plastids and starch disappear during tumor formation, leaving empty host cells around areas of abundant sporulation. The lipid composition of the tumor tissue on maize ears has actually been characterized in some detail because these galls are an edible delicacy in Mexico (known as cuitlacoche or "corn truffle"). Based on nutritional analysis, LCFA are abundant components of cuitlacoche, particularly oleic and linoleic acid followed by
linolenic and palmitic acid (Hawke et al., 1974; Valverde and Paredes-Lopez, 1993; Vanegas et al., 1995). Oleic, linoleic and palmitic acid are also known to be predominant fatty acid species in teliospores of *U. maydis* (Gunasekaran et al., 1972). Of course, tumor tissue represents a mixture of plant and fungal material and it is therefore difficult to separate the relative contributions to fatty acid content.

### 3.3.2. Possible Roles for Lipid Signaling in Fungal Morphogenesis and Plant Defense

Part of our investigation into the role of *mfe2* considered the question of whether a defect in fatty acid metabolism would influence the morphological transition leading to infectious hyphae. This question was motivated by our previous observations that exogenous lipids trigger the dimorphic switch from budding to hyphal growth in culture (Klose et al., 2004). Notably, I found that *mfe2* was required for filamentation in response to some but not all fatty acids. For example, palmitic acid (C16:0) triggered the formation of unbranched hyphae that resembled the mating filaments and the initial infectious dikaryons that usually form on a leaf surface during early stages of infection. Oleic acid and linoleic acid induced highly branched filaments in wild-type cells but the *mfe2* mutants formed filaments with very short branches on oleic acid and did not respond morphologically to linoleic acid. Unlike wild-type strains, growth of the mutants was limited on both fatty acids. These results suggest that exogenous linoleic acid may have a specific signaling role, either directly or after processing, to form derivatives such as oxylipins.

Precedent for the regulation of fungal morphogenesis by fatty acids and oxylipins comes from studies on sporulation, secondary metabolite production and sexual development in filamentous fungi (Goodrich-Tanrikulu et al., 1998; Kock et al., 2003; Tsitsigiannis and Keller, 2006; Tsitsigiannis et al., 2005a; Tsitsigiannis et al., 2005b; Tsitsigiannis et al., 2005c; Tsitsigiannis et al., 2004a; Tsitsigiannis et al., 2004b). For example, the influence of oxylipins has been studied in detail in *Aspergillus nidulans*, and it has been shown that hydroxylated oleic, linoleic and linolenic acids constitute an endogenous mixture of oxylipin hormones (psi factors) that control the timing and balance of meiotic and mitotic spore development (Calvo et al., 2001; Champe et al., 2002).
1987; Tsitsigiannis et al., 2005a; Tsitsigiannis et al., 2004a; Tsitsigiannis et al., 2005b; Tsitsigiannis et al., 2005c; Tsitsigiannis et al., 2004b). More recent work revealed that mutants defective in the fatty acid oxygenases for psi factor production (ppo genes) had reduced production of the mycotoxin sterigmatocystin, reduced peanut seed colonization, and increased penicillin production (Tsitsigiannis and Keller, 2006). With regard to potential oxylipin metabolism, a polypeptide (Sspl) with similarity to linoleate diol synthase has been found to be abundant in U. maydis teliospores, and a function in the mobilization of storage lipids has been proposed (Huber et al., 2002). However, deletion of the ssp1 gene does not result in an obvious phenotype. It is possible that U. maydis responds to derivatives of fatty acids, and that mfe2 and β-oxidation may be required to produce specific intermediates that are further modified to produce signaling lipid/fatty acid molecules. It may also be the case that the loss of mfe2 has an indirect affect on morphogenesis by influencing cellular fatty acid composition as demonstrated by our chemical analyses. Clearly, further investigation of the specificity of signaling among fatty acids and derivatives such as oxylipins is needed.

The mfe2 mutation impaired symptom development during infection but did not completely abolish fungal growth within the plant. In general, biotrophic fungi like U. maydis evade plant defense mechanisms by camouflage or by suppression, or actively defend themselves by mechanisms such a detoxification of host metabolites (Schulze-Lefert and Panstruga, 2003). It is possible that an enhanced defense response occurs upon infection by mfe2 mutants because of the altered chemical environment caused by the metabolic defect in the pathogen. That is, a change in lipid composition or signaling could trigger a defense response through activation of jasmonic acid signaling and the defense responses of the salicylic acid-dependent pathway. Lipids clearly have an impact on the plant defense response through a variety of mechanisms (Shah, 2005). For example, fatty acids play an important role in modulating signaling between the salicylic acid (SA)- and jasmonic acid (JA)-dependent defense pathways and recent studies have uncovered an important role for lipids also in the activation of systemic acquired resistance (Kachroo et al., 2003; Maldonado et al., 2002; Nandi et al., 2004). In general, little is known about the defense response of maize to infection by U. maydis. Basse (2005) recently described transcriptional changes for maize genes related to metabolism
and development in response to *U. maydis* infection, as well as putative defense responses of the host. Evidence was obtained to suggest that *U. maydis* is capable of suppressing a defense response based on the observation that a weakly proliferative mutant triggered expression of the pathogenesis related gene PR-1. These observations suggest that it may be informative to examine defense-related gene expression during infection with *mfe2* mutants, although I favor the hypothesis that a nutritional defect results in reduced growth *in planta*.

In summary, I have demonstrated the importance of a β-oxidation function for growth on fatty acids in culture and for biotrophic infection. However, many additional questions remain about the importance of lipid metabolism in *U. maydis* during infection including the potential role of lipases, the extent of lipid utilization and the specificity of potential signals from fatty acids and their derivatives. In addition, the genome sequence suggests that the fungus may also have a mitochondrial β-oxidation system and the contribution of this system to infection remains to be explored.

3.4. Materials and Methods

3.4.1. Growth Conditions

*U. maydis* strains were grown overnight at 30°C on both potato dextrose agar or broth (PDA or PDB; Difco), or complete medium (CM) (Holliday, 1974). For selection of transformants, 250 micrograms of hygromycin B per mL was added to CM. To characterize the morphological response and to determine growth rate in lipids and fatty acids, 1X10^6 mL^{-1} of PDB-grown overnight cells were washed with sterile water and added to 5 mL of minimal medium (MM) (Holliday, 1974) or spotted in a range of cell concentrations on MM agar supplemented with one of the following carbon sources: glucose, corn oil, butyric, caproic, lauric, myristic, oleic, linoleic, erucic, arachidic, arachidonic, or jasmonic acid; all added to a concentration of 1%. (all fatty acids were purchased from Sigma). The cells were grown at 30°C for 5 days with (liquid MM) or without (MM agar) shaking at 250 rpm. The growth of wild-type and mutant strains was determined by cell counts with a hemacytometer.
3.4.2. Strains, Deletion Constructs and Transformation Procedures

The DNA sequence of the mfe2 gene (Um00150) was originally obtained from the U. maydis genomic sequence at Broad Institute (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/). Additional sequence information (Um10038) came from the MIPS’s U. maydis genome annotation project (http://mips.gsf.de/genre/proj/ustilago/). A PCR overlap strategy was used to generate the Δmfe2::hygB′ deletion construct (Davidson et al., 2002). The Δmfe2::hygB′ was designed to delete the entire open reading frame of the mfe2 gene. From genomic DNA, a 655 bp 5′flanking region and a 550 bp 3′ flanking region were amplified using primers MFE2P1 (5′-AGTTTCGAGTCGGTGCGT-3′) and MFE2P2 (5′-AACTGTGCTTCAATCGCTGCGGTATGCGGCTGTGAGTTGA-3′), and primers MFE2P5 (5′-TAGCACACGACTCACATCTGCGAGTGCATGGTGGTGAGTGA-3′) and MFE2P6 (5′-TGAGCGTTGCAATCGTG-3′), respectively. The 2.7kb hygromycin resistance marker was amplified using primers MFE2P3 (5′-TCAACTCACAGCCGCATACCGCAGCGATTGAAGCACAGTT-3′) and MFE2P4 (5′-ATCTCACCCACCAGTCAGCGATGTGCTGTGGTGCTA-3′) from the plasmid DNA pIC19RHL. The three fragments were combined by an overlapping PCR reaction using primers MFE2P1 and MFE2P6. The 4 kb overlap PCR product generated the Δmfe2::hygB′ construct, which was cloned into pCR2.1 (Invitrogen). The plasmid containing the deletion construct was transformed into E. coli strain DH10B (Bethesda Research Laboratories).

The deletion strains, a1b1 Δmfe2::hygB′ and a2b2 Δmfe2::hygB′ were generated by biolistic transformation (Toffaletti et al., 1993) of a1b1 (521) and a2b2 (518) strains (Holliday, 1961). Transformants were screened by colony PCR using a U. maydis-specific primer outside the construct MFE2CR (5′-TCTCGCACCAGTCAATCCTG 3′) and hygB-specific primer HYGBL (5′-ATCAGTTCGGAGACGCTG-3′). Gene deletion was also confirmed by DNA blot analysis using genomic DNA and blots prepared and hybridized by standard methods (Sambrook et al., 1989). Two independent deletion mutants in each wild-type strain were selected for all subsequent phenotypic analyses to
confirm that the deleted gene is responsible for the observed phenotypes. Representative data from analyses of all of the deletion strains is shown in the results section of this chapter.

3.4.3. RNA Isolation and Northern Analysis

Cells were grown overnight in 5 mL MM supplemented with glucose (1%) and transferred to MM supplemented with either caproic, oleic or linoleic acid (all added to 1%) and grown at 30°C at 250 rpm for 6 hrs. RNA was isolated as described previously (Schmitt et al., 1990). RNA blot preparation and hybridization was performed using standard methods (Sambrook et al., 1989). PCR was used to amplify the 150 bp DNA fragment as a hybridization probe using primers MFE2NP1 (5' AGAGCACCGTCTTCATTCG-3') and MFE2NP2 (5'-TGTGAAGCGCACCTTGATG-3'). The probe was labeled with $^{32}$P by random priming (ReadiPrime™ II Oligolabeling kit, Amersham Pharmacia Biotech).

3.4.4. Sequence Analysis

Gene prediction, protein alignments and sequence analysis were done using the programs BLAST (Altschul et al., 1997), CLUSTAL W (Thompson et al., 1994), and Pfam (Bateman et al., 2002).

3.4.5. Microscopy and Staining Procedures

Fluorescent brightener 28 Calcofluor white (Sigma, F3543) was used to visualize cell walls and 1 μL of a 20 μg/mL solution was added directly to 5 μL of cell culture spotted on a slide. Nile Red (Sigma N3013) was used to visualize lipid bodies by adding 1μL of a 0.1mg/mL solution in 100% acetone directly to 5 μL of cell culture, incubated for 5 minutes and observed using a FITC filter. Nile red stains intracellular lipids that localize in lipid bodies (Kimura et al., 2004). Fungal proliferation in a plant tissue was observed in epidermal peels generated from maize leaves at one, four, and seven days post inoculation. The thin layers of plant cells were placed on a 30 μL drop of water with
3 μL of Fluorescent brightener 28 calcofluor white (Sigma, F3543) for microscopic observation. Cross sections of tumor tissue collected from infected mature plants 14 and 20 days post inoculation were generated using a razor blade. The cross sections were placed on a 30 μL drop of water with 3 μL of Fluorescent brightener 28 calcofluor white (Sigma, F3543) for microscopic analysis. Cells were observed using a Zeiss Axioplan 2 fluorescence microscope with differential interference contrast (DIC) optics or UV fluorescence to observe cells stained with Calcofluor or Nile Red. Images were captured with a DVC camera and processed with Northern Eclipse imaging software and Adobe Photoshop 7.

For electron microscopy, cells were prefixed by the addition of glutaraldehyde (2.5%) to cultures grown for 18 hr either on glucose (1%) or oleic acid (1%) with shaking at 250 rpm. The cells were harvested by centrifugation for 10 min at 16,100 X g at 20°C, resuspended in 50 mM phosphate buffer (pH 6.8) containing 3% glutaraldehyde to an OD600 of 10, and fixed for 24 h at room temperature. For specific lipid staining, the cells were post-fixed with 2% OsO4 in 200 mM imidazole buffer (pH 7.5) for 1 h. After washing with 100 mM imidazole buffer (pH 7.5), the cells were dehydrated in a graded ethanol series in the following order: 50, 70, 90, and 100% (vol/vol). For transmission electron microscopy (TEM), the cells were embedded in Spurs resin and 70-nm-thick sections were cut with a Leica Ultracut E ultramicrotome, and stained with 2% uranyl acetate for 14 minutes and lead citrate for seven minutes. Sections were mounted on 200-mesh grids and examined with a Hitachi H7600 TEM. A random sample of cells was examined in three separate cultures from each type of medium.

3.4.6. Lipid Extraction and Fatty Acid Analysis

Cells were grown in 50 mL MM supplemented either with glucose (1%) or oleic acid (1%) at 30°C at 250 rpm for 5 days. The cells were harvested by centrifugation and washed twice with hexane (Sigma, H9379). To extract the intracellular lipids, the cells were sonicated and 2 mL methanolic-KOH was added to saponify lipids for 2hr at 80°C, and then washed by adding 1 mL water and 2 mL hexane. 400 μL concentrated HCl and 2 mL hexane were added to the bottom phase to extract free fatty acids (FFA).
Heptadecanoic acid (C17:0) (Sigma, H3500) was added to the extracted lipids as an internal standard. FFA were converted into fatty acid methyl esters (FAME) by adding diazomethanol and incubating at room temperature for 4 hr. The FAME were analyzed by gas chromatography mass spectrometer (GC/MS) Agilent Technologies 5975 Inert XL MS Detector with 6890N GC and 7683 B Series autosampler equipped with a capillary GC column Agilent HP-5MS 5% phenyl methyl siloxane.

3.4.7. Mating and Virulence Assays

Strains were tested for their ability to mate by the production of white aerial hyphae during mating reactions on charcoal-containing CM (Holliday, 1974). For virulence assays, mating cultures of 1X10^7 cells mL^-1 (grown on PDB overnight at 30°C with shaking at 250 rpm) generated by crossing the strains in the following combinations was used to infect maize plants: 521 (albl) X 518 (a2b2), 521 (albl) X a2b2 Δmfe2::hygB', 518 (a2b2) X albl Δmfe2::hygB' and albl Δmfe2::hygB' X a2b2 Δmfe2::hygB'. For seedling infections, one-week-old maize plants (Golden Bantam) were inoculated by injecting approximately 100 μL of mating cultures per plant. After 14 days, plants were scored for disease symptoms using the following ratings: (1) chlorosis and pigment production; (2) small leaf tumors; (3) small stem tumors; (4) large stem tumors; and (5) plant death. Approximately 100 plants for each combination of strains were scored for disease symptoms. For mature plant infections, two to three-month-old maize plants were inoculated by injecting approximately 2 mL of mating cultures into the silk channels of developing cobs. The infections were repeated four times. The production of teliospore within tumors was examined after 14 and 20 days.

3.4.8. Accession Numbers

Sequence data from this article can be found in the EMBL Nucleotide Sequence Submission (EMBL), http://www.ebi.ac.uk, and GenBank, National Center for Biotechnology Information (GenBank), http: //www.ncbi.nlm.nih.gov, data libraries under accession number XP_756297.
3.5. Tables

Table 3.1  Fatty acid profiles of total internal lipids extracted from *U. maydis* wild-type (*a2b2*) and *mfe2* mutant (Δ*mfe2* *a2b2*) strains grown either on glucose or oleic acid

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Glucose a2b2</th>
<th>Glucose Δmfe2 a2b2</th>
<th>Oleic acid a2b2</th>
<th>Oleic acid Δmfe2 a2b2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% ± SD</td>
<td>% ± SD</td>
<td>% ± SD</td>
<td>% ± SD</td>
</tr>
<tr>
<td>C14:0</td>
<td>1 ± 0.2</td>
<td>n/d</td>
<td>t e</td>
<td>n/d</td>
</tr>
<tr>
<td>C16:0</td>
<td>23 ± 0.5</td>
<td>t e</td>
<td>17 ± 2.1</td>
<td>t e</td>
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<tr>
<td>C16:1</td>
<td>1 ± 0.0</td>
<td>1 ± 0.5</td>
<td>1 ± 0.5</td>
<td>1 ± 0.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>5 ± 1.3</td>
<td>t e</td>
<td>4 ± 0.5</td>
<td>t e</td>
</tr>
<tr>
<td>C18:1</td>
<td>29 ± 2.4</td>
<td>79 ± 3.3</td>
<td>20 ± 3.8</td>
<td>90 ± 0.2</td>
</tr>
<tr>
<td>C18:2</td>
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</tbody>
</table>

a The table shows data from three independent experiments.
b % of total fatty acids
c Not detected
d Internal lipids were extracted from cells grown in minimal medium supplemented with appropriate carbon source for 20 hrs. Fatty acids were analyzed by gas chromatography.
e Found in trace amounts
Table 3.2 Pathogenicity of mfe2 mutants

<table>
<thead>
<tr>
<th>Cross or strain</th>
<th>No. of plants producing anthocyanin</th>
<th>No. of plants with tumors</th>
<th>Total no. of plants infected</th>
<th>% of plants with tumors</th>
<th>Disease score $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a1b1 \times a2b2$</td>
<td>12</td>
<td>87</td>
<td>99</td>
<td>88</td>
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<tr>
<td>$a1b1 \times \Delta mfe2 a2b2$</td>
<td>16</td>
<td>95</td>
<td>106</td>
<td>90</td>
<td>3.4</td>
</tr>
<tr>
<td>$\Delta mfe2 a1b1 \times a2b2$</td>
<td>10</td>
<td>82</td>
<td>92</td>
<td>89</td>
<td>3.6</td>
</tr>
<tr>
<td>$\Delta mfe2 a1b1 \times \Delta mfe2 a2b2$</td>
<td>75</td>
<td>28</td>
<td>103</td>
<td>27</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$ Table shows combined data from four independent experiments. The pattern of the disease symptom development shown in the table is representative of a pattern observed in each independent experiment.

$^b$ The disease score is calculated as the sum of disease symptoms ratings divided by the total number of infected plants scored for symptoms.
3.6. References


4. HAD1 IS REQUIRED FOR TELIOSPORE GERMINATION AND MAY PLAY A ROLE IN EARLY STAGES OF IN PLANTA FUNGAL DEVELOPMENT IN USTILAGO MAYDIS

4.1. Introduction

The phytopathogen *Ustilago maydis* is an excellent experimental organism to study plant-microbe interactions. Specifically, the fungus provides a genetically tractable system to investigate the relationship between metabolism and pathogenic development in a biotrophic fungus. This is because the early stages of fungal development can be initiated in culture where two haploid yeast-like cells of compatible mating type are able to exchange pheromone signals and mate to produce a dikaryotic filament capable of infecting host tissue (Banuett and Herskowitz, 1989; Böcker et al., 1995; Gillissen et al., 1992; Holliday, 1974; Kamper et al., 1995; Kronstad and Staben, 1997; Puhalla, 1969; Snetselaar et al., 1996). After penetration, the fungus is completely dependent on the host plant, maize (*Zea mays*), for further development and completion of its life cycle. Several morphological changes take place during fungal development within host tissue that cannot be achieved in culture, with the main change being the formation of a filamentous cell type that is capable of sustained proliferation (Banuett and Herskowitz, 1996) Snetselaar and Mims, 1992, 1994). Once inside a host, *U. maydis* induces tumor production, and the fungal filaments exhibit highly branched filamentous growth and extensive proliferation within the tumor tissue. Within the tumors, the fungus literally replaces the contents of plant cells with its own biomass. By the end of the life cycle, the filaments differentiate to produce diploid spores and the tumors are covered with only a thin layer of plant cell walls. These features of morphogenesis and pathogenicity in *U. maydis* are governed by well conserved signaling pathways including the cAMP/protein kinase A (PKA) pathway and the mitogen-activated protein kinase (MAPK) cascade (Andrews et al., 2000; Banuett and Herskowitz, 1994; Barrett et al., 1993; Durrenberger et al., 2001; Durrenberger et al., 1998; Gold et al., 1994; Gold et al., 1997; Hartmann et al., 1996; Mayorga and Gold, 1999).
The nutrient rich environment of host cells supports extensive proliferation of *U. maydis* during the course of an infection, but the nutritional requirements of the fungus *in planta* are still not understood. Previously, we reported that *U. maydis* can utilize lipids as a sole carbon source (Klose et al., 2004). Consistent with this observation, triglyceride lipase activity was detected in culture, and it was found that a PKA mutant that is unable to respond to fatty acids also exhibits less extracellular triglyceride lipase activity (Klose et al., 2004). Intriguingly, the β-oxidation pathway in peroxisomes of *U. maydis* is required for full symptom development and therefore contributes to the virulence of the fungus (Klose and Kronstad, 2006). Specifically, a mutation in the gene *mfe2* encoding the multifunctional β-oxidation enzyme reduced *in planta* proliferation and tumor production leading to attenuated virulence. Furthermore, lipids in the form of triglycerides or fatty acids serve not only as carbon sources for *U. maydis*, but also as signals to initiate yeast-to-filamentous growth transition needed for invading plant tissue (Klose et al., 2004).

The breakdown of fatty acids is important in the metabolism, development, and pathogenicity of fungi. For example, *Aspergillus* spp. were shown to utilize lipids as growth substrates (Kawasaki et al., 1995; Maggio-Hall and Keller, 2004; Mellon et al., 2002), and lipase activity was linked to virulence in *Aspergillus* spp. and *Fusarium gramineum* (Voigt et al., 2005). Lipid catabolism occurs via the β-oxidation pathway in which fatty acids are oxidized by a series of enzyme steps releasing acetyl-CoA and an acyl-CoA shortened by two carbons, which can undergo additional cycles of β-oxidation. In general, mammalian β-oxidation of long-chain fatty acids occurs predominantly in peroxisomes, while medium- and short-chain fatty acids undergo β-oxidation in the mitochondria (reviewed in Eaton et al., 1996; Wanders et al., 2001). In contrast, in *Saccharomyces cerevisiae* and other yeasts, fatty acids are metabolized entirely in peroxisomes (reviewed in (Hiltunen et al., 2003). Recently Maggio-Hall and Keller (2004) presented evidence that the filamentous fungus *A. nidulans* has both peroxisomal and mitochondrial β-oxidation systems. In mitochondria, typically four individual reactions take place for β-oxidation, each catalyzed by a separate enzyme. However, it has been shown that there is a second, long-chain β-oxidation pathway in the mitochondria of mammals, where the second and third steps are catalyzed by a
‘trifunctional enzyme’ (Uchida et al., 1992). In general, electrons removed during oxidation are passed to oxygen via the electron transport chain, yielding ATP (Ferarman, 1988). In addition, mitochondrial β-oxidation disorders in humans have only been recognized relatively recently and these produce a diverse array of clinical presentations, often resulting in death (Coates and Tanaka, 1992; Wood, 1999). Mouse models for short-chain acyl-CoA dehydrogenase (SCAD) and 3-hydroxyacyl-CoA dehydrogenase (HAD) deficiencies have been described (Guerra et al., 1998; O'Brien et al., 2000).

In this study, I show that *U. maydis*, in contrast to *S. cerevisiae*, possesses mitochondrial β-oxidation, based on the prediction of genes for mitochondrial enzymes in the *U. maydis* genome and the characterization of the mitochondrial 3-hydroxyacyl-CoA dehydrogenase (*hadl*). Because of the requirement for peroxisomal β-oxidation in the virulence of *U. maydis* (Klose and Kronstad, 2006), the mitochondrial β-oxidation gene *hadl* was characterized to explore further the importance of the β-oxidation during developmental stages of the fungus in the plant host. It was found that the mutation in *hadl* gene that blocks mitochondrial β-oxidation resulted in severe attenuation of disease symptoms in maize seedlings. Additionally, the ability of teliospores, collected from the mutant infection of the host plant, to germinate was impaired. The mutation in *hadl* also resulted in an inability to grow on short-chain (SCFA) and medium-chain fatty acids (MCFA) as expected. Fungal growth also was attenuated after the addition of arabinose into the fatty acid medium even for the wild-type strains, suggesting that these fatty acids inhibited the growth of *U. maydis*. Overall, this study has relevance for understanding metabolic aspects of the interactions between plants and fungal pathogens.

### 4.2. Results

**The *U. maydis* genome possesses genes encoding putative mitochondrial β-oxidation enzymes**

It has been proposed that β-oxidation in yeast fungi, such as *S. cerevisiae*, *Yarrowia lipolytica* and *Candida tropicalis* takes place exclusively in peroxisomes (Hiltunen et al., 1992; Kunau et al., 1995; Kurihara et al., 1992; Smith et al., 2000). Intriguingly, the analysis of the genome sequence performed as part of this study revealed
that *U. maydis* has candidate genes encoding the monofunctional enzymes thought to be involved in the β-oxidation of fatty acids in mitochondria. These genes were predicted to be present based on a strong similarity to known proteins involved in mitochondrial β-oxidation in mammalian systems. Specifically, there were five genes (Um01466, Um10665, Um06185, Um01049 and Um00694) predicted to encode the acyl-CoA dehydrogenase known to catalyze the first step in mitochondrial β-oxidation in mammalian systems. Interestingly, six genes (Um04973 and Um01433, Um02097, Um03071, Um02762 and Um03158) were predicted to encode the enoyl-CoA hydratase catalyzing the second reaction. One gene (Um01099) was predicted to encode 3-hydroxyacyl-CoA dehydrogenase (HAD), the enzyme catalyzing the third reaction, and three genes (Um03298, Um01843 and Um03571) were predicted to encode 3-ketoacyl-CoA thiolase, the enzyme catalyzing the last reaction of mitochondrial β-oxidation. In addition, no homologs to the putative *U. maydis* mitochondrial β-oxidation enzymes were found in a search of the *S. cerevisiae* genome. This agrees with previous findings that β-oxidation occurs exclusively in the peroxisomes of *S. cerevisiae* (Hiltunen et al., 1992). However, there were homologs found in the filamentous fungi *A. nidulans* and *Neurospora crassa*. The predicted mitochondrial monofunctional enzymes present in the genome suggest that *U. maydis* possesses both peroxisomal and mitochondrial β-oxidation.

The gene (*hadl*) predicted to encode 3-hydroxyacyl-CoA dehydrogenase in the genome was chosen for subsequent study to investigate the importance of the mitochondrial β-oxidation in morphogenesis and pathogenesis of *U. maydis*. Choosing *hadl* for the study, the only gene predicted to encode HAD in the genome, avoided potential problems with redundancy that might occur with the multiple genes encoding other enzymes in the mitochondrial β-oxidation pathway. Originally, the amino acid sequence of the human 3-hydroxyacyl-CoA dehydrogenase enzyme (Q16836) was used to identify the *hadl* gene in *U. maydis* genome (http://www.broad.mit.edu/annotation/genome/ustilago_maydis). The closest fungal homologue of Had1 was a predicted protein in *Cryptococcus neoformans* (EAL19129, 54% identity and 71% similarity), and the closest homologues from organisms other than fungi were characterized proteins in *C. elegans* (NP509584, 50% identity and 66%
similarity) and human (Q16836, 53.3% identity and 66% similarity). Overall, the HAD proteins are highly conserved in their amino acid sequence throughout different species (Figure 4.1). The hadl gene of U. maydis encodes a 344 amino acid (aa) protein comprised of only one exon. In general, 3-hydroxyacyl-CoA dehydrogenase is an enzyme with a double-domain structure (Birktoft et al., 1987). The amino-terminal domain (3HCDH_N; pfam 02737), which comprises approximately the first 200 residues (from 39 to 232 aa in the predicted U. maydis polypeptide sequence), is responsible for binding the NAD cofactor. The carboxyl-terminal domain (3HCDH; pfam 00725) comprises the remaining 105 residues (from 234 to 338 aa in the predicted U. maydis polypeptide sequence). Target IP was used to identify a predicted signal peptide at the amino-terminus that could function to localize the protein to the mitochondrion (Emanuelsson et al., 2000).

4.2.1. hadl transcript levels are regulated by growth in fatty acids

To explore the role of the hadl gene in U. maydis, and its potential function in fatty acid metabolism, I examined hadl transcript levels during growth of the fungus on various fatty acids and glucose. The expression of hadl in fatty acids was assessed using Northern analysis (Figure 4.2). Specifically, transcription of hadl was induced by growth in short-chain caproic acid (C6) and medium-chain myristic acid (C14) as expected. Interestingly, growth in long-chain oleic acid (C18:1) or linoleic acid (C18:2) also induced transcription of hadl at comparable levels to the transcription induced by caproic and myristic acid. These LCFA have also been shown to induce transcription of the multifunctional enzyme (mfe2) in peroxisomes (Klose and Kronstad, 2006). Thus, LCFA in U. maydis might be first oxidized in peroxisomes to shorten the chains, and then transported to mitochondria for further processing to produce energy, as shown in mammalian systems. Furthermore, growth in all of the fatty acids tested resulted in higher hadl transcript levels in the wild-type strain relative to the cells grown on glucose (Figure 4.2). Overall, these results indicate that hadl transcription is regulated by fatty acids independent of their carbon-chain length.
Figure 4.1 Sequence alignment of the *U. maydis* Had1 polypeptide with 3-hydroxyacyl-CoA dehydrogenases (HADs) from different organisms.

The *U. maydis* Had1 sequence was compared to the proteins from *C. elegans* (NP509584), human (Q16836), *Mus musculus* (Q61425) and *Thermoanaerobacter tengcongensis* MB4 (AAM23824). The alignment revealed a high conservation of these enzymes across the species. Identical residues are highlighted in yellow.
Figure 4.2 Transcript levels for the *had1* gene in cells grown in fatty acids that differ in their chain length.

Total RNA was isolated from wild-type cells (strain 518; *a2h2*) grown in glucose or one of the fatty acids: caproic, myristic, oleic or linoleic. (A) The RNA blot hybridized with *had1* probe. (B) RNA blot stained with 0.04% methylene blue to reveal the rRNA bands and demonstrate equal loading of the lanes.
4.2.2. **Generation of hadl mutant strains**

To investigate the function of hadl, a targeted gene deletion was performed in which the complete coding region of the hadl gene (1035 nt) was replaced with a 1.8 kb nourseothricin gene cassette (Figure 4.3A, and Materials and Methods). The gene deletion construct was initially introduced into the a2b2 wild-type strain (518) of *U. maydis*. The nourseothricin-resistant transformants were selected and analyzed by colony PCR (data not shown) and Southern blot (Figure 4.3B). The wild-type hadl allele was present on a 4 kb Clal SacI restriction enzyme fragment, while the gene deletion was confirmed by the presence of a 5 kb Clal SacI fragment. The deletion of hadl in the albl wild-type strain (521) of the opposite mating type was generated by the sexual cross of the albl wild-type strain with the a2b2 hadl::nat' strain in corn seedlings. The haploid progeny from the germinated diploid teliospores produced from the cross were analyzed for their mating types and for deletion of hadl. The nourseothricin-resistant colonies were selected and first analyzed by mating to identify strains with the albl mating type and next by Southern blot (Figure 4.3B and data not shown). Overall, eight mutants in each mating type (albl and a2b2) were collected from total of 55 nourseothricin resistant colonies that were selected for further analysis. For the albl strain, the wild-type hadl allele was present on a 8 kb Clal NdeI fragment and the deletion was confirmed by the presence of a 2 kb Clal NdeI fragment (Figure 4.3B). Two independent deletion mutants in the a2b2 strain and three mutants in the albl mating-type strains from the sexual cross were selected for all subsequent phenotypic analyses.

4.2.3. **Deletion of hadl eliminated growth on short-chain and medium-chain fatty acids**

The hadl gene was predicted to encode HAD enzyme activity that would be required for growth on short-chain and medium chain fatty acids. Therefore, the strains deleted in hadl were assessed for their ability to grow in medium supplemented with short-chain (SCFA, caproic acid), medium-chain (MCFA, lauric and myristic acids) and also long-chain (LCFA; oleic acid) fatty acids as a sole carbon source (Figure 4.4A).
Figure 4.3 Targeted gene deletion of the *U. maydis* had1 gene.

(A) Restriction map showing the orientation of the *had1* locus and the structure of the deletion construct. The ~1 kb coding region of *had1* (red arrow) was replaced with a 1.8 kb nourseothricin resistance cassette (blue arrow) generated using overlap PCR (Davidson et al., 2002). The left and right arms represent 5' and 3' flanking regions surrounding the *had1* gene. (B) DNA blot analysis of *had1::sat* transformants. Genomic DNA was prepared from the wild-type strains *a1b1* and *a2b2* and the deletion strains (number 28 and 34 in *a2b2*, and 24, 31 and 41 in *a1b1*) in both genetic backgrounds. Strains were analyzed using *ClaI/SacI* and *ClaI/NdeI* restriction enzymes in the *a2b2* and *a1b1* strain backgrounds, respectively. The blot was hybridized with a 600 bp PCR-amplified probe whose position is indicated in (A). Homologous integration of the deletion construct is indicated by the hybridization of a 5 kb and 2 kb fragment in *a2b2* and *a1b1*, respectively, and the absence of a signal for 4 kb (in *a2b2*) and 8 kb (in *a1b1*) wild-type fragments.
As expected, the mutation in this gene resulted in an inability to grow on SCFA and MCFA. Specifically, the mutant strains did not grow on caproic, lauric or myristic acid. The mutants also exhibited reduced growth on oleic acid but grew well on non-fatty acid carbon sources, such as glucose or arabinose (Figure 4.4B). Interestingly, caproic and lauric, but not myristic fatty acids also limited the growth of the wild-type strains, probably due to an inhibitory effect on growth.

To further explore the possible inhibition of growth by SCFA and MCFA, I measured the growth of the wild-type and mutant strains in arabinose minimal medium supplemented with caproic, lauric or myristic acid (i.e., fatty acids of differing chain length) (Figure 4.4B). While addition of myristic acid into the arabinose medium supported additional growth of the wild-type strain, the addition of caproic or lauric acids attenuated the growth of the wild-type strains, again indicating that these fatty acids inhibited the growth of U. maydis. The mutant strains were unable to grow on any of these fatty acids even in the presence of arabinose indicating that growth was completely inhibited. Caproic and lauric acids were previously found to inhibit the growth of A. nidulans (Maggio-Hall and Keller, 2004).

4.2.4. Myristic acid does not induce filamentation in hadl mutants

Previously, we showed that fatty acids can serve as signaling molecules to trigger the dimorphic switch from budding to filamentous growth in U. maydis (Klose et al., 2004). To determine whether mitochondrial β-oxidation plays a role in this transition, the hadl mutants were assessed for their ability to respond morphologically to fatty acids (Figure 4.5). The wild-type and mutant strains were grown in minimal medium supplemented with SCFA (caproic acid), MCFA (lauric or myristic acids) or LCFA (oleic acid) as a sole carbon source. In addition, non-fatty acid carbon sources, such as glucose or arabinose, were also added to the minimal medium as a sole carbon source for a control. As expected, both the wild-type and mutant strains exhibited budding growth in glucose or arabinose medium. Interestingly, while the hadl mutants produced wild-type-like filaments in LCFA (data not shown), they did not respond by filamentous growth to SCFA or MSFA (Figure 4.5).
Figure 4.4 Cellular growth of *had1* mutants on short and medium chain fatty acids.

(A) The total numbers of the wild-type (*α2h2*, black bars) and mutant (*Δhad1 α2h2*, white bars) cells is shown for cultures supplemented with fatty acids differing in their carbon chain length: SCFA (caproate), MCFA (laurate and myristate) or LCFA (oleate) as a sole carbon source. (B) *U. maydis* growth inhibition by SCFA and MCFA. The total numbers are shown for cells grown in glucose or arabinose as a sole carbon source, or the fatty acids together with arabinose. The bars represent the average number of cells from three independent experiments based on cell counts at day five.
The lack of a response on caproic, lauric or myristic acid may be due to the inability of the *hadl* mutants to grow on these fatty acids. To ensure that the lack of a response in these fatty acids is due to growth limitation, arabinose (i.e., representing a non-repressing sugar) was added into the medium containing fatty acids. Interestingly, in the presence of arabinose, the mutants responded to caproic and lauric, but not myristic fatty acid by growing as filaments (Figure 4.5). Therefore, the *hadl* gene is not required for the morphological transition in caproic and lauric acid, however it is required specifically for the response to myristic acid. Interestingly, selective response to specific fatty acids depending on their chain length and saturation state was previously observed for the mutants defective in peroxisomal β-oxidation in *U. maydis* (Klose and Kronstad, 2006).

### 4.2.5. Intracellular lipid accumulation in *hadl* mutants

To explore whether mitochondrial β-oxidation would influence the distribution of intracellular lipids, I compared the accumulation of lipid bodies within cells of the wild-type and the mutant strains grown on SCFA (caproic acid) and MCFA (myristic acid) as a sole carbon source. The accumulation of internal lipids in lipid bodies was determined by staining the cells with Nile red (Figure 4.6). Large numbers of lipid bodies accumulated in wild-type cells grown on both caproic and myristic acids. The lipid bodies were visible throughout the entire cells and varied in size depending on the fatty acid chain length. Particularly, cells grown on caproic acid produced large lipid bodies and cells on myristic acid produced small bodies. In contrast, the mutant strains produced very few small lipid bodies when grown on caproic acid. Interestingly, only a diffused fluorescence and no accumulation of lipid bodies was detected in the mutants grown on myristic acid, which correlates with their inability to respond to this fatty acid.

### 4.2.6. Deletion of *hadl* does not impair mating ability in *U. maydis*

To determine the effect of the *hadl* deletion on mating, *hadl* mutants were co-spotted with either compatible wild-type strains (*a1b1* and *a2b2*) or compatible mutant strains (Δ*hadl a1b1* and Δ*hadl a2b2*) on charcoal-containing mating medium.
<table>
<thead>
<tr>
<th></th>
<th>$a2b2$</th>
<th>$\Delta hdl a2b2$</th>
</tr>
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<td>myristate + arabinose</td>
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<td><img src="image16" alt="Image" /></td>
</tr>
</tbody>
</table>

**Figure 4.5 Fatty acid-induced filamentation in $hldl$ mutant strains.**

Cellular morphology is shown for the wild-type ($a2b2$) and mutant ($\Delta hdl a2b2$) strains in response to glucose, arabinose and SCFA (caproate) and MCFA (laurate and myristate) with or without arabinose. The cells were visualized by differential interference contrast optics (DIC, left) and by epifluorescence after staining cell walls with calcofluor (right). Scale bar = 10 μm.
Figure 4.6 Intracellular lipid accumulation in hadl mutant strains.

*U. maydis* wild-type (*a2b2*) and mutant (Δ*hadl a2b2*) strains grown on SCFA (caproate) or MCFA (myristate) as a sole carbon source. The internal lipids accumulated in lipid bodies were stained using the lipid-specific fluorescent dye Nile red and visualized using epifluorescence. The fungal cells produced large (arrowhead) to small (arrow) lipid bodies that varied in number depending on carbon source. Scale bar = 10 μm.
Mating filaments, visible as white aerial hyphae growing on the agar surface, were produced when the mutants were co-spotted with the wild-type cells, indicating a positive mating reaction (data not shown). These mating reactions were comparable to those observed when compatible wild-type strains were mated. The hadl mutants were also able to produce aerial hyphae when co-spotted with compatible hadl mutant strain, indicating that hadl was not required for mating.

The morphology of filaments produced during the mating reaction of compatible hadl mutants was also assessed to investigate whether hadl influences morphogenesis during the early stages of dikaryon production. The aerial hyphae were scrapped off the surface of the mating plates and analyzed microscopically. No differences in the morphology of the cells from the compatible mutant mating reaction were observed when compared to the cells from the compatible wild-type mating reaction (data not shown). In both cases, the cells were found in different stages known to take places during mating reaction of U. maydis. Particularly, the filamentous dikaryon, conjugation tubes and some budding cells were observed. Overall, these results show that Had1 was not required for cell fusion and production of initial dikaryotic filaments capable of infecting plant tissue.

4.2.7. hadl mutants have reduced virulence in maize seedlings

Typical disease symptoms in a plant inoculated with U. maydis begin with chlorosis and anthocyanin pigment production followed by tumor production. To assess the role of hadl in pathogenicity, the hadl mutants were inoculated in combination with compatible wild-type strains (e.g., Δhadl a1b1 X a2b2 and a1b1 X Δhadl a2b2) or as mixtures of compatible hadl mutant strains (Δhadl a1b1 X Δhadl a2b2) into maize seedlings (Table 4.1). Given the positive mating ability of the hadl mutants, I expected the mutants to cause disease on maize seedlings. However, I observed that the deletion of hadl resulted in reduced virulence. Specifically, the hadl mutants were still able to infect the seedlings and induce tumors, but the size of tumors and the frequency of tumor production were reduced. For example, only approximately 20% of the mutant-inoculated
plants developed tumors compared to 90% of the plants that developed tumors when inoculated with wild-type strains. (Table 4.1) Strikingly, a dramatic reduction in the incidence of death in the plants infected with the mutants was also observed when compared to the wild-type infections. In parallel with the reduction in plant death, the number of plants with no symptoms also increased. Interestingly, in many cases plants exhibited only chlorosis, and no further symptoms such as the anthocyanin pigmentation that takes place prior to tumor production were observed. While the hadl mutants were still able to cause full range of the disease symptoms, the decrease in tumor induction and the incidence of plant death is intriguing. Furthermore, the deletion in hadl resulted in reduced virulence even when the hadl mutants were inoculated with compatible wild-type strains ($\Delta$hadl a1b1 X a2b2 and a1b1 X $\Delta$hadl a2b2). In these cases, approximately 60% of the plants produced tumors, which reflects a 30% reduction in tumor incidence when compared to the wild-type inoculations. This suggests that the deletion of only one allele is enough to cause the virulence defect (haploinsufficiency) and that both alleles are necessary for full activity of the hadl gene product during fungal development in planta. These findings provide evidence that mitochondrial $\beta$-oxidation may be crucial during specific stages of in planta fungal development for *U. maydis*.

4.2.8. **Hadl is required for teliospore germination**

The ability of a teliospore to undergo germination is crucial for *U. maydis* to initiate an infection and begin sexual development. The germination of the diploid spore gives rise to haploid sporidia, that are able to recognize a mating partner upon the exchange of pheromone signals, and to produce conjugation tubes. The conjugation tubes eventually fuse to produce the infectious dikaryon able to penetrate plant tissue and thus initiate plant infection. Thus, no infection can take place without germination of teliospores. Therefore, I investigated whether the deletion of hadl affects spore germination. Teliospores were collected from tumors developed on infected maize seedlings and were incubated to allow germination. The germination of teliospores was determined microscopically after 16 hrs and 24 hrs of incubation. To evaluate the ability of teliospore to germinate, the whole area of a microscope slide containing agar with
incubated teliospores collected from the mutant tumors was examined in three separate occasions for each experiment. Germination of teliospores from the wild-type cross (i.e., wild-type tumors) was observed after 16 hrs of incubation, but only a small number of the teliospores had started germinating (Figure 4.7). Furthermore, mature melanized teliospores were isolated in large numbers from the wild-type tumors. In contrast, teliospores collected from plants inoculated with the hadl mutants (i.e., mutant tumors) did not germinate even after 24 hrs of incubation (Figure 4.7). The teliospores were incubated for additional 24 hr to confirm that the germination was not just delayed. Even after 48 hr, there was no germination observed in the hadl mutant teliospores (data not shown). 300 teliospores were counted to assess the germintation from each cross in three independent samples. The cross sections of tumors from maize seedlings infected with hadl mutants indicated reduced number of mature teliospores (data not shown). There were relatively few mature teliospores isolated from the tumors from the mutant infections and most of the teliospores were still not fully developed at the time of harvest. These results suggest that the mitochondrial β-oxidation function is required for successful teliospore germination. However, more experiments are underway to precisely quantify the germination ability of teliospores produced from the hadl mutant infections.

4.3. Discussion

In this study I show, based on the genome sequence analysis, that U. maydis possesses a mitochondrial β-oxidation system, and I characterized the hadl gene that is predicted to encode a mitochondrial 3-hydroxyacyl-CoA hydratase. As expected, hadl was required for growth on short-chain and medium-chain fatty acids, and hadl transcript levels were regulated by fatty acids. hadl was required specifically for myristic acid-induced filamentation and accumulation of lipid bodies during growth on this fatty acid. For pathogenicity, hadl was not required for the initial production of the infectious dikaryon during mating, but was required for disease symptom development in maize seedlings and germination of teliospores. Specifically, deletion of hadl results in a dramatic reduction in the incidence of death in plants and an increase in plants with no symptoms.
Figure 4.7 Had1 is required for teliospore germination.

Teliospores were harvested 14 days after inoculation of maize seedlings with the wild-type (albl X a2b2) and mutant (Δhad1 albl X Δhad1 a2b2) crosses. A germinating teliospore (i.e., black with thick outer layer that does not stain with calcofluor) is shown from the wild-type cross. The spore has an extended promycelium from which haploid progeny are generated (i.e., stained with calcofluor) after 16- and 24-hr incubation period. A teliospore from the mutant cross did not germinate even after 24 hrs of incubation. The cells were visualized by differential interference contrast optics (DIC, top panel) and by epifluorescence after staining cell walls with calcofluor (bottom panel). Scale bar = 10 μm.
These findings provide evidence that the mitochondrial β-oxidation may be crucial during teliospore germination and important during initial stages of in planta fungal development in U. maydis.

It has been proposed that fungal β-oxidation takes place solely in peroxisomes because all β-oxidation activities are carried out in peroxisomes in S. cerevisiae and other yeasts such as C. tropicalis and Y. lipolytica (reviewed in Trotter, 2001). However, genome analysis revealed that U. maydis has candidate genes encoding enzymes of both peroxisomal and mitochondrial β-oxidation. For the mitochondrial system, there appear to be multiple genes encoding monofunctional acyl-CoA dehydrogenases, enoyl-CoA hydratases and thiolases (catalyzing the first, second and fourth reactions, respectively), and only one copy of the 3-hydroxyacyl-CoA dehydrogenase (Hadl). Based on genetic analysis, the Hadl enzyme appears to be capable of oxidizing short- and medium-chain fatty acids. While deletion of hadl resulted in a restricted growth on long-chain fatty acids (oleic acid, C18), the capacity of this pathway to actually degrade substrates as long as oleic acid remains to be determined. Some 3-hydroxyacyl-CoA dehydrogenases in mammals are active on a wide range of fatty acids (short- to long-chain) (Fong and Schulz, 1977; Liang et al., 2001). Baltazar et al. (1999) suspected the presence of β-oxidation in mitochondrion in A. niger. However, it has only just recently been demonstrated that A. nidulans is capable of both peroxisomal and mitochondrial β-oxidation (Maggio-Hall and Keller, 2004).

Previously, I described the construction and analysis of mutants defective in the peroxisomal β-oxidation multifunctional enzyme Mfe2 in U. maydis (Klose and Kronstad, 2006; and Chapter 3 in this thesis). Taken together, the phenotypes of the mitochondrial and peroxisomal β-oxidation pathway mutants in U. maydis grown on fatty acids of differing chain lengths suggest that both pathways work together to accomplish full growth on these carbon sources. While the function of the Mfe2-dependent pathway appears essential only for very long-chain fatty acids (Klose and Kronstad, 2006) and the Hadl-dependent pathway appears to be necessary for short- and medium-chain fatty acids, both pathways seem to play a role in catabolism of long-chain fatty acids. Peroxisomal β-oxidation in mammals functions primarily to shorten very long fatty acids (C22 or higher) and a variety of branched-chain fatty acids encountered in the diet
(reviewed in Wanders et al., 2001), and little activity is exhibited toward short-chain acyl-CoAs in vitro (Vanhove et al., 1993). In addition, medium length acyl-CoAs are transported from the peroxisome to the mitochondrion (reviewed in Ramsay, 2000). Such cooperative functions of β-oxidation systems leading to the ability to metabolize a range of different fatty acids may also exist in U. maydis. In conclusion, based on the predicted genes for mitochondrial enzymes in the U. maydis genome and the evidence that the mitochondrial β-oxidation exists in other fungi, as demonstrated recently in A. nidulans (Maggio-Hall and Keller, 2004), I propose that U. maydis contains both peroxisomal and mitochondrial β-oxidation.

Deletion of the had1 gene revealed the importance of mitochondrial β-oxidation in pathogenicity of U. maydis. The had1 gene was required for full symptom development in planta and germination of teliospores in vitro. The first stage of the life cycle of a phytopathogenic fungus is generally the germination of a spore and the development of structures required for host penetration. Because of the paucity of external nutrient sources on the plant surface, fungi must rely on endogenous storage compounds as a source of energy. Deletion of had1 completely abolished teliospore germination and pathogenicity tests showed that the number of plants that developed disease symptoms was significantly decreased. The lack of symptom development could indicate a defect early during the penetration stage. In U. maydis, the appressoria-like structures (i.e., swellings at the tip of the infectious dikaryon) penetrate through the plant surface and a functional interface between the fungus and the host is established (Snetselaar and Mims, 1993, 1994). It has been proposed that lipid catabolism is critical during early stages of fungal development (reviewed in Solomon et al., 2003). Some of the evidence comes from genomic studies in Blumeria graminis showing that lipid catabolism persists throughout the germination and penetration phases. It has also been shown that glyoxylate cycle in T. yalundae and M. grisea, and peroxisome biogenesis in Colletotrichum lagenarium, are important during the penetration phase (Bowyer et al., 2000; Kimura et al., 2001; Wang et al., 2003). Both of these processes probably involve peroxisomal β-oxidation of fatty acids. Interestingly, our studies on peroxisomal β-oxidation in U. maydis suggest that this pathway is important during later phases of fungal development, such as in planta proliferation and teliospore differentiation (Klose and Kronstad, 2006). Based on this study mitochondrial β-oxidation
function seems to be important during the early infectious phases, which involve germination and perhaps penetration of host tissue. This would allow \textit{U. maydis} to metabolize alternative carbon sources such as fungal storage lipids for germination, and to utilize fungal storage lipids for infection-related morphological development (penetration).

During plant infection with \textit{hadl} mutants, the size of tumors and the rate of tumor production were reduced, and many plants exhibited only chlorosis and no further symptom development. The reduction in symptom development could indicate a defect in the early events of penetration, but also in the establishment of infection such that less severe symptoms developed. Many biotrophic fungi develop specialized feeding structures (haustoria) used for the uptake of nutrients and, potentially, signal exchange during infection (Birch et al., 2006; Mendgen and Hahn, 2002). \textit{U. maydis} lacks such structures, but a prolonged interaction zone is established during the initial intracellular growth of infecting hyphae (Snetselaar and Mims, 1992, 1994). During this time, the translocation of various compounds into the host may take place that may be necessary to initiate and/or establish prolonged filamentous growth within plant tissue. Therefore, it is possible that \textit{U. maydis} must rely on stored intracellular lipids (i.e., lipid catabolism) to produce such compounds and to provide basic metabolic needs at least during the initial phase of growth in the plant. In addition, cross sections of tumors from maize seedlings infected with \textit{hadl} mutants indicated reduced number of mature teliospores, suggesting that Had1 may play a role during teliospore differentiation. However, because of the decreased number of plants that developed symptoms during infections, the low number of mature teliospores may just reflect the defect of the \textit{hadl} mutants during the early stages of infection, thus influencing the subsequent stages of fungal development. In addition, Had1 was not required for cell fusion and production of initial dikaryotic filament capable of infecting plant tissue. Thus the mutants may have a specific defect during the early stages of growth \textit{in planta}.

It is intriguing that \textit{hadl} mutant infections resulted in a dramatic decrease in the incidence of plant death. The reduction in plant death could indicate that host defense reactions may be successful in limiting the fungal growth of \textit{hadl} mutants, perhaps due to less vigorous growth during infection. Alternatively, an enhanced defense response
could occur upon infection by had1 mutants because of the altered chemical environment caused by the metabolic defect in the pathogen. Similar phenotypes were also observed during infections by mfe2 mutants defective in peroxisomal β-oxidation (Klose and Kronstad, 2006). It is possible that Had1 may play a role in allowing U. maydis to bypass or suppress plant defense reactions or reprogram the metabolism of the host to allocate resources to the fungus during the initial stages of infections. Recently, Basse (2005) has demonstrated that U. maydis is capable of suppressing a defense response in maize based on the observation that a weakly proliferative mutant triggered expression of the pathogenesis related gene PR-1.

In summary, this study addressed the role of the mitochondrial β-oxidation of fatty acids in plant-fungal interactions to contribute to our understanding of the role that lipid metabolism may play in the virulence of phytopathogenic fungi. Mitochondrial β-oxidation appears to be crucial during teliospore germination, penetration and initial stages of in planta fungal development in U. maydis. Additional experiments, such as the generation of double mutants deleted in both mitochondrial and peroxisomal β-oxidation will be helpful to further understand the importance of β-oxidation in virulence of U. maydis.

4.4. Material and Methods

4.4.1. Growth conditions

Fungal strains were grown in potato dextrose broth (PDB), potato dextrose agar (PDA), or on complete medium agar (CM) as described previously (Holliday, 1974). To characterize the morphological response and to quantitate growth in fatty acids, 1X10^6 mL^-1 of PDB-grown overnight cells were washed once with sterile water and added to 5 mL of minimal medium (MM; Holliday, 1974) supplemented with glucose, caproic, lauric, myristic or oleic acid (Sigma) as a sole carbon source (all added to a concentration of 1%). The cells were grown at 30°C for 5 days with shaking at 250 rpm. The extent of growth was determined by cell counts with a hemacytometer. Transformants were initially grown on double complete medium (DCM) with 1 M sorbitol and then streaked onto CM agar containing 100 μg mL^-1 nourseothricin (Werner BioAgents) for antibiotic
selection. *Escherichia coli* strain DH10B (Bethesda Research Laboratories) was used for transformation by electroporation and was grown as previously described (Sambrook et al., 1989).

### 4.4.2. Strains, deletion constructs and transformation procedures

The DNA sequence of the *hadl* gene (Um01099) was originally obtained from the *U. maydis* genomic sequence that was completed at the Broad Institute (http://www.broad.mit.edu/annotation/fungi/ustilago_maydis/). Recently, the Munich Institute for Protein Sequences (MIPS) completed a *U. maydis* genome annotation project and the annotated version of the *hadl* gene can now be found at [http://mips.gsf.de/genre/proj/ustilago/](http://mips.gsf.de/genre/proj/ustilago/). The Δ*hadl::sat* deletion construct was generated using a PCR overlap strategy (Davidson et al., 2002) and was designed to include 0.6 kb of genomic sequence of the 5’ upstream sequence (left arm) and 1 kb of the 3’ downstream (right arm) sequence from the *hadl* gene surrounding a nourseothricin selectable marker (*satl*; Gold, Gold et al., 1994) to ensure a complete deletion of the coding region. The left arm and right arms were amplified using primers Had1P1 (5’-CTGCTTGTGCCTTGACATT-3’) and Had1P2 (5’-AACTGTGCTTCAATCGCTGCCCAGCGTCCAATGATTGTGA-3’), and primers Had1P5 (5’-TTGCAGAACTCGCTGGTAGTCGCTATCCTTCTGATCGTCT-3’) and Had1P6 (5’-GGTGTTGGAGTGCAGGTAAT-3’), respectively. The 1.8 kb nourseothricin resistance marker was amplified from the plasmid pSatll2 using primers Had1P3 (5’-TCACAATCATGGACGCTGGGCAGCGATTGAAGCACAGTT-3’) and Had1P4 (5’-AGACGATCAAGGAGATAGCGACTACCACGCGAGGTCTGCA-3’). The three fragments were combined by an overlapping PCR reaction using nested primers Had1P1n (5’-CTGCTTGTGCCTTGACATT-3’) and Had1P6n (5’-GGAGTGCAGGTAATCACGAA-3’). The 3.4 kb overlap PCR product generated the Δ*hadl::sat* construct, which was cloned into pCR2.1 (Invitrogen) generating pCRHad1. The pCRHad1 plasmid containing the deletion construct was transformed into *E. coli* strain DH10B (Bethesda Research Laboratories).
The deletion strains \(a2b2 \Delta{\text{had}1::sat}^{\prime}\) and \(a1b1 \Delta{\text{had}1::sat}^{\prime}\) were generated by biolistic transformation (Toffaletti et al., 1993) of strain 518 (mating type \(a2b2\); Holliday, 1961) and by a sexual cross with strain 521 (\(a1b1\)) (Holliday, 1961). For the sexual cross, teliospores harvested from maize seedlings inoculated with the cross of the \(a1b1 \times a2b2\) strains were extracted from tumor tissue and 200 \(\mu\)L of the teliospore extract was streaked on PDA and incubated at 30°C for 24 hrs. To select for \(\Delta{\text{had}1::sat}^{\prime}\) deletion strains, the haploid progeny were grown on CM agar containing nourseothricin. To determine mating type of the \(\Delta{\text{had}1::sat}^{\prime}\) progeny, the strains were tested for their ability to mate with \(a1b1\) and \(a2b2\) strains. Transformants and deletion strains from the sexual cross were screened by colony PCR using a \(U.\ maydis\)-specific primer Had1P1 outside the construct (5'- CTGCTTGTGCGCTTGACATT -3') and a sat1-specific primer PSatT (5'-GCTTCCGAAGATGGCTCTGT-3'). Gene deletion was also confirmed by Southern blot analysis. Two independent deletion mutants in the \(a2b2\) strain and three mutants in the \(a1b1\) mating-type strains from the sexual cross were selected for all subsequent phenotypic analyses to confirm that the deleted gene is responsible for the observed phenotypes. Representative data from analyses of all of the deletion strains is shown in the results section of this chapter. Gel electrophoresis, restriction enzyme digestion and DNA blot hybridization were performed using standard procedures (Sambrook et al., 1989).

4.4.3. RNA isolation and northern analysis

Fungal cells were grown overnight in 5 mL MM supplemented with glucose (1%) and transferred to MM supplemented with either glucose, caproic, myristic, oleic or linoleic acid (all added to 1%) and grown at 30°C at 250 rpm for 6 hrs. RNA was isolated as described previously (Schmitt et al., 1990). PCR was used to amplify a 455 bp DNA fragment spanning from 109 bp to 564 bp in the exon of the \(\text{had}1\) gene as a hybridization probe using primers Had1Fw (5'-CAGAACAAGGACGTGAGAAA-3') and Had1Rv (5'-GTGGAAGCCTCCGAATAGTTs-3'). The probe was labeled with \(^{32}\text{P}\) by random priming (ReadiPrime™ II Oligolabeling kit, Amersham Pharmacia Biotech).
Standard procedures were followed for RNA blot preparation and hybridization (Sambrook et al., 1989).

4.4.4. **Sequence analysis**

Gene prediction, protein alignments and sequence analysis were done using the programs BLAST (Altschul et al., 1997), CLUSTAL W (Thompson et al., 1994), Pfam (Bateman et al., 2002), SignalP s3.0 (Bendtsen et al., 2004) and TargetP 1.1 (Emanuelsson et al., 2000).

4.4.5. **Mating and pathogenicity assays**

Fungal cells were spotted on double complete medium with 1% activated charcoal (DCM-C) for mating tests (Day and Anagnostakis, 1971; Holliday, 1974). Strains were tested for the production of aerial hyphae during mating reactions as previously described (Day and Anagnostakis, 1971). For *U. maydis* pathogenicity assays, 7-day old maize seedlings were inoculated, and disease symptoms were evaluated as described (Kronstad and Leong, 1989). The pathogenicity tests were performed using the following strain combinations: 521 (albl) X 518 (a2b2), 521 (albl) X a2b2 Δhad::satl', 518 (a2b2) X albl Δ had::satl' and albl Δ had::satl' X a2b2 Δ had::satl', where two independent hadl mutants in a2b2 background (28-1and 34-1) and three hadl mutants in albl background (24-2, 31-2 and 41-2; generated from the sexual cross) were used to inoculate the seedlings. Approximately 100 plants for each combination of strains were scored for disease symptoms in three independent experiments.

4.4.6. **Teliospore isolation and germination**

Teliospores were isolated from tumors collected from inoculated maize seedlings 14 days post inoculation. Tumors were first sterilized in 10% bleach for 30 seconds and washed twice in sterile dH2O. Sterilized tumors were ground in 20ml of 1.5% CuSO4.5H2O using sterile mortar and pestle. Ground tumor tissue (containing teliospores) was filtered through cheesecloth and incubated overnight at room
temperature. Teliospore suspensions were centrifuged and washed twice in sterile dH2O, and 200μl of the suspension was spread on PDA plates (containing a microscope slide) and incubated at 30°C for approximately 16 to 24 hrs. The microscope slide was cut out from the agar and 1μl of a 20 μg mL⁻¹ solution of fluorescent brightener 28 Calcofluor White (F3543, Sigma) diluted in 5μl of sterile dH2O was placed directly on a coverslip prior to observations. Germination of teliospores was observed at two time points, at 16 and 24 hrs. Teliospores were isolated from two independent experiments and germination was observed in three separate experiments.

4.4.7. Microscopic analysis

Cell walls were visualized by staining with 1μl fluorescent brightener 28 Calcofluor White (20 μg mL⁻¹; F3543, Sigma) added directly to 5μl of cell culture on a microscope slide. To observe the production of mating filaments, filaments were scraped off of mating medium and resuspended in sdH2O plus 1 μL of a 20 μg mL⁻¹ fluorescent brightener 28 Calcofluor White (F3543, Sigma). Lipid bodies within yeast-like and filamentous cells grown in fatty acids were visualized by staining with a Nile Red solution (0.1mg mL⁻¹ in 100% acetone) (N3013; Sigma) by adding 1μL directly to 5μl cell culture on a microscope slide followed by observation using a FITC filter (excitation at 450-490 nm). Cells were observed using a Zeiss Axioplan 2 fluorescence microscope with differential interference contrast (DIC) optics or UV fluorescence to observe cells stained with Calcofluor. Images were captured with a DVC camera and processed with Northern Eclipse imaging software and Adobe Photoshop 7.

4.4.8. Accession Numbers

Sequence data from this chapter can be found in the EMBL Nucleotide Sequence Submission (EMBL), http://www.ebi.ac.uk, and GenBank, National Center for Biotechnology Information (GenBank), http: //www.ncbi.nlm.nih.gov, data libraries under accession number XP757246. U. maydis gene numbers described above refer to the MIPS database.
4.5. Tables

Table 4.1 Pathogenicity of *had1* mutants

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<th>No. of plants producing anthocyanin</th>
<th>No. of plants with tumors</th>
<th>Total no. of plants infected</th>
<th>% of plants with tumors</th>
<th>Disease score $^b$</th>
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<td>22</td>
<td>109</td>
<td>20</td>
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</table>

$^a$ Table shows combined data from three independent experiments. The pattern of the disease symptom development shown in the table is representative of a pattern observed in each independent experiment.

$^b$ The disease score is calculated as the sum of disease symptoms ratings divided by the total number of infected plants scored for symptoms.
4.6. References


5. PHOSPHOLIPASE A2 ATTENUATES THE DISEASE SYMPTOM DEVELOPMENT DURING INFECTION OF USTILAGO MAYDIS

5.1. Introduction

_Ustilago maydis_ is a biotrophic fungal pathogen that requires a host plant to sporulate and complete its life cycle. The plant tissue provides both nutrients and signals for fungal growth and development. Recent analysis revealed that the _U. maydis_ genome possesses genes encoding for secreted proteins that cluster together and that, in many cases, are _Ustilago_-specific (Kämper et al., 2006). Mutations in some of these genes cause changes in virulence. Therefore, it has been proposed that these genes may have evolved to function in specific aspects of the biotrophic life style. A variety of secreted proteins, such as hydrolytic enzymes (proteases, phospholipases and lipases) have been shown to contribute to the virulence of many pathogenic microorganisms (Chen et al., 1997a; Chen et al., 1997b; Hube, 1998; Hube et al., 2000; Ibrahim et al., 1995; Leidich et al., 1998; Saffer et al., 1989; Walker et al., 1983).

Extracellular phospholipases, secreted during the course of infection, are believed to be virulence factors for many pathogenic bacteria and protozoa such as _Clostridia_ species, _Listeria monocytogenes_, _Pseudomonas_ species, _Staphylococcus aureus_, _Mycobacterium tuberculosis_ and _Toxoplasma gondii_ (reviewed in Ghannoum, 2000). There is some evidence supporting a role for extracellular phospholipases as virulence factors in experimental fungal infections. For example, the human pathogenic fungus _Cryptococcus neoformans_ secretes a phospholipase enzyme that demonstrates phospholipase B (PLB), lysophospholipase hydrolase and lysophospholipase transacylase activities, and that has been proposed to be a virulence factor for the fungus (Chen et al., 1997a, 1997b; Cox et al., 2001). The role of extracellular phospholipase as a potential virulence factor has also been demonstrated for other pathogenic fungi, including _Candida albicans_ (Leidich et al., 1998). Because phospholipases are secreted, it has been postulated that these enzymes assist in the penetration of phospholipid-rich host barriers such as membranes. Invasion of host cells by most pathogens requires penetration and damage of the cell membrane, which is mediated by either physical or enzymatic means, or a combination of both. Phospholipases such as the pH-activated phospholipase A2 of
Helicobacter pylori (Berstad et al., 2002) are likely to be involved in the membrane disruption processes (Waite, 1996), because phospholipids represent one of the major chemical constituents of the host cell envelope. However, it is still unclear how extracellular phospholipases serve as virulence factors in fungal infections.

Phospholipases are a diverse group of lipolytic enzymes that share the ability to hydrolyze one or more ester linkages in phospholipids. Although all phospholipases target phospholipids as substrates, they vary in the site of action on the phospholipid molecule, their function and mode of action, and their regulation (Waite, 1996). Phospholipases were given identifying letters A, B, C, and D to indicate the specific bond targeted in the phospholipid molecule (Figure 5.1). Phospholipases function in various roles ranging from the digestion of nutrients to the formation of bioactive molecules. This diversity of function indicates that phospholipases can serve auxiliary functions (e.g. in virulence), and can be critical to central cellular processes. For example, the continual remodeling of cell membranes requires the action of one or more phospholipases (Schmiel and Miller, 1999).

I initiated a project to examine the role of genes encoding phospholipases as part of a larger program to explore the involvement of lipid metabolism in the lipid-induced filamentous response (Klose et al., 2004) and virulence in U. maydis. Previously, one of the phospholipase A2 genes, lip1, was identified in our group in a subtractive hybridization screen as being highly expressed in a strain mutated in hgll (Mario Moniz de Sa, unpublished). Hgll is thought to be a direct target of PKA that is required for mating, and is thought to act as a negative regulator of budding growth (Durrenberger et al., 2001). Deletion of lip1 has a negative influence on filamentous growth during mating, demonstrated by the fact that the hgll lip1 double mutants restored the ability of hgll mutants to mate. There is evidence in other fungi that fatty acid products of phospholipase A2 activity influence the activity of adenylyl cyclase (AC) (Resnick and Tomaska, 1994). Therefore, Lip1 may function in a feedback loop to regulate AC by maintaining high cAMP levels to promote budding growth. However, the lip1 mutants caused disease symptoms at the wild-type levels (Mario Moniz de Sa, unpublished).
Figure 5.1 Sites of action of various phospholipases.

The arrows show ester bonds that are cleaved by specific phospholipases depending on their site of action. PLA1 = phospholipase A1; PLA2 = phospholipase A2; PLB = phospholipase B; PLC = phospholipase C; PLD = phospholipase D.
Phospholipase A2 (PLA2) hydrolyses the fatty acyl ester bond at the sn-2 position of the glycerol moiety (Figure 5.1). The action of PLA2 results in the accumulation of free fatty acids and 1-acyl lysophospholipid. PLA2 plays an important role in signal transduction in mammals, in particular in generation of proinflammatory mediators such as prostaglandins and leukotrienes, and in membrane remodeling. Several subtypes of mammalian PLA2 have been described. These are divided in four main groups according to their function, localization, and calcium dependency. Cytosolic phospholipase A2 associates with natural membranes in response to physiological increases in Ca2+, and selectively hydrolyses arachidonoyl phospholipids to initiate cell-to-cell signaling (van den Berg et al., 1995). This enzymatic activity cleaves off arachidic acid from the membranes in mammalian cells, often initiates signal transduction, and is regulated by the state of the cell activation. Platelet-activating factor acethylhydrolase (PAF-AH), a subfamily of phospholipase A2, is responsible for inactivation of platelet-activating factor through cleavage of an acetyl group (i.e., at the second position of glycerol in bioactive phospholipids), releasing lyso derivatives of phospholipid substrates and short fatty acids (Dennis, 1997; Derewenda and Derewenda, 1998). In animal systems, several structurally unrelated types of PLA2 have been described (Dennis et al., 1995). The release of arachidonic acid leading to the synthesis of inflammatory lipid mediators (eicosanoids) requires the activity of different PLA2 isoforms in distinct cell types (Balboa et al., 1996). In plants, there is indirect evidence for the coordinated involvement of three types of phospholipases (A, C and D) in defense signaling (Chapman, 1998; Munnik et al., 1998). Activation of PLA2 has been shown in response to auxin (Paul et al., 1998), wounding (Narvaez-Vasquez et al., 1999) and elicitors. In addition, soybean cells have also been found to respond to treatment with bacterial and fungal elicitors by PLA2 activation (Chandra et al., 1996). Secreted and membrane-bound PLA2 activity has also been described in bacteria, fungi and protozoa (Ghannoum, 2000; Matoba et al., 2002; Waite, 1996), and reported to be related to pathogenesis in some microorganisms (Schaller et al., 2005).

In this study, I describe the relevance of one gene encoding a putative phospholipase A2 (Lip2, predicted to belong to the PAF-AH family) to the virulence of U. maydis. I found that deletion of lip2 increases the virulence of the fungus. Infection
with the mutant resulted in the enhanced production of disease symptoms in both seedlings and mature maize plants. In addition, the mutation in lip2 did not impair mating reactions, or the ability of the teliospores to germinate. Lip2 was also not required for the filamentation of cells that is triggered by lipids.

5.2. Results

5.2.1. Genes coding for putative phospholipases

To explore the possible role of phospholipases in virulence of *U. maydis*, I first annotated the available genomic sequence for the presence of specific types of phospholipase enzymes potentially secreted by the fungus. There were two putative phospholipase A1 (PLA1) genes, Um02255 and Um05659, in the genome and both are of the phosphatidic acid-preferring PLA1 (PA-PLA1) type. PA-PLA1 specifically hydrolyzes phosphatidic acid and contains the DDHD domain (PF02862). Interestingly, there was no match in the genome to PLA1 (EC 3.1.1.32), the acyl hydrolase enzymes with broad substrate specificity. One gene for a putative cytosolic phospholipase A2 (PLA2; EC3.1.1.4) (Um05871) and two genes for putative platelet activating factor hydrolases (PAF) (Um00133 and Um01927) were found in the genome. This enzyme contains a lysosphospholipase (lpl) catalytic domain and a C2 domain (Pfam00168).

One gene, Um01035, was predicted to code for a putative phospholipase B (PLB; (synonyms: lysophospholipase, lysophospholipase-transacylase)) enzyme (EC3.1.1.5) based on a strong similarity to PLB proteins from *S. cerevisiae*, *C. neoformans*, *N. crassa* and *S. pombe*. It contained a predicted lysosphospholipase catalytic domain. There are other putative lysosphospholipases (Lyso-PLB) in the genome but these lack the lysosphospholipase domain. The predicted genes include Um00130 with an esterase/lipase/thioesterase domain and a phospholipase/carboxylesterase domain, Um02599 with an esterase/lipase/thioesterase domain, and Um01007 with ankyrin and asparaginase/glutaminase domains.

There are two putative genes predicted to encode for phospholipase C (PLC), Um02982 and Um01865 (Meldrum et al., 1991). They both contain the PLC catalytic domain X and Y, two regions that together form a TIM barrel-like structure containing
the active site residues. The Um02982 gene also contains the EF-hand calcium-binding motif, where calcium binding leads to the activation or inactivation of target proteins (Rhee and Choi, 1992a, 1992b).

Three potential phospholipase D (PLD) genes were found in the genome (Um00370, Um01120, and Um06066) and all contain the PLD/transphosphatidylase domain. Um00370 has strong similarity to the *S. cerevisiae* SPO14 gene (responsible for meiosis and spore formation) (Ella et al., 1996; Rose et al., 1995; Waksman et al., 1996). The SPO14-like PLD enzyme is highly conserved among fungal species. Um01120 has high similarity to phosphatidylglycerophosphate (PG-P) synthase PEL1/PGS1 from *S. cerevisiae*. PG-P synthase catalyzes the synthesis of PG-P from CDP-diacylglycerol and sn-glycerol 3-phosphate in the biosynthesis of cardiolipin (CL) (Chang et al., 1998). Um06066 encodes a conserved hypothetical protein with no significant homology to other proteins.

5.2.2. *lip2* gene identification in the *U. maydis* genome

The amino acid sequence of the previously characterized *lip1* gene (Mario Moniz de Sa, unpublished) was originally used to search for other possible PLA2 genes in the genome. Based on the genome analysis, the *lip2* gene was predicted to encode the second platelet-activating factor acetylhydrolase PLA2. Lip1 and Lip2 proteins share 32% identity and 46.6% similarity. The *lip2* gene encodes a predicted polypeptide of 829 amino acids, consisting of only one exon with one putative domain (amino acids 48 to 344) for platelet-activating factor acetylhydrolase plasma/intracellular isoform II (PAF-AH_p_II) at the N-terminus, and a predicted hydrolase fold (HAD superfamily) at the C-terminus (amino acids 502 to 698). Lip2 protein shows highest sequence similarity to a hypothetical protein in the fungus *Gibberella zeae* (26% identity and 44% similarity, accession number XP_380370).

5.2.3. Construction of *lip2* mutants

A targeted gene deletion strategy was used to delete the coding region of the *lip2* gene by replacing the entire open reading frame with a 3.8 kb gene cassette conferring
resistance to the antibiotic hygromycin B. Deletion of the gene was confirmed by colony PCR and Southern blot analysis (Figure 5.2). Two independent restriction enzyme digests (Ndel and SalI) and two different probes (probeL and probeR) were used to verify a lip2 deletion allele. The wild-type lip2 allele was present on a 3.1 kb SalI restriction enzyme fragment and a 5.3 kb Ndel fragment, while the gene deletion was confirmed by the presence of a 2.7 kb SalI fragment and a 1.6 kb Ndel fragment. The deletion of lip2 was performed in two wild-type strains of opposite mating types (a1 b1 and a2 b2) to allow mating and pathogenicity analysis to investigate the role of PLA2 in virulence (see Materials and Methods). Two independent mutant strains from each mating background were selected and used for subsequent phenotypic analyses.

5.2.4. lip2 mutants respond to fatty acids by growing filaments

We have previously shown that lipids and specifically fatty acids trigger the dimorphic switch from budding to filamentous growth in U. maydis wild-type strains (Klose et al., 2004). This fatty acid-induced filamentation is dependent on the functions of both the cAMP/PKA and MAP kinase signaling pathways. Therefore, I wanted to determine whether the lip2 gene plays a role in filamentous growth induced by fatty acids, and thus whether the phospholipase A2 activity contributes to the dimorphic switch. Mutant strains deleted in lip2 were grown in a liquid minimal medium supplemented with glucose, corn oil, palmitic acid or oleic acid as a sole carbon source (Figure 5.3). As expected, the mutant cells exhibited yeast-like budding phenotype when grown in glucose medium. However, when grown in a presence of either of the lipid or fatty acid carbon sources, the cells underwent the morphological transition from budding to filamentous growth. Therefore, the phospholipase A2 enzyme encoded by lip2 does not seem to play a role in morphogenesis in U. maydis in response to lipids and fatty acids.
Figure 5.2 Targeted gene deletion of lip2 gene in U. maydis.

(A) Schematic representation and restriction enzyme maps of the wild-type lip2 locus and the deletion construct. The deletion construct contains a 2.7 kb hygromycin resistance
replacing the 2.5 kb coding region of lip2 and the right and left arms of lip2 generated using PCR overlap (Davidson et al., 2002). The left and right arms represent the 5' flanking region and the 3' flanking region of the lip2 gene. (B) DNA gel blot analysis of lip2::naf transformants. Genomic DNA from the wild-type strains 518 (a2b2) and 521 (albl) (labeled as WT), the lip2::naf mutant transformants (3, 6, 12, 13, 14, 15, 16 in albl; 5, 6, 9, 10, 20 in a2b2), and the ectopic integration transformant 8 (in a2b2), was digested with either SalI (top) or NdeI (bottom) and processed for gel blot analysis. The appearance of a band size of 2.7 kb in the SalI digest and 1.6 kb in the NdeI digest was diagnostic of the correct gene replacement event. The wild-type lip2 allele is present on a 3.1 kb SalI restriction enzyme fragment and a 5.3 kb NdeI fragment.

5.2.5. Lip2 is not required during the early stages of pathogenic development including mating and penetration

Mating of compatible haploid cells takes place on the plant surface during the initiation of infection. During the mating reaction, the haploid cells produce conjugation tubes that grow between the mating partners and eventually fuse to form the infectious dikaryon. The dikaryotic filament penetrates the plant surface and invades host tissue. To determine whether lip2 plays a role in the initial stages of infection, I performed mating and penetration analyses. For mating reactions, the strains of opposite mating type were mixed on charcoal-containing agar plates (Figure 5.4). A positive mating reaction is indicated by the presence of a white colony appearance due to aerial hyphae production on the surface during mating (Figure 5.4A). The lip2 mutants were able to mate when tested with compatible wild-type mating partners (albl and a2b2) and also when combined with other lip2 deletion strains of the opposite mating type (Δlip2 albl and Δlip2 a2b2). Therefore, mating was unaffected in lip2 mutants. Microscopic observations of the mating filaments were also performed to determine whether the mutation had an effect on morphology or the rate of filament production during mating (Figure 5.4B). The wild-type strains produced many long unbranched filaments, conjugation tubes, and some cells remained yeast-like. Similar cellular morphologies were observed in the mutant mixtures: filaments, conjugation tubes, and yeast-like cells.
Figure 5.3 Cellular morphology in response to oils and fatty acids.

Wild-type and lip2 mutant strains were grown on minimal medium supplemented with glucose, corn oil, palmitic or oleic acid as sole carbon source. The cells were visualized by differential interference contrast optics (DIC). Scale bar = 10 μm.
Figure 5.4 Response of lip2 mutants to mating.

(A) Colony morphology resulting from the mating reaction on charcoal-containing agar. The reaction between wild-type strains 518 (albl) and 521 (a2b2) in the center shows the white aerial hyphal growth indicative of a positive mating reaction. Mating reactions between compatible wild-type and mutant strains and compatible mutant strains resulted in positive mating reactions similar to wild-type. (B) Cellular morphology of the mating filaments produced during wild-type and mutant mating interactions. The lip2 mutants produced mating filaments similar to wild-type strains. The cells were visualized by differential interference contrast optics (DIC). Scale bar = 10 μm.
Therefore, the deletion of *lip2* did not influence the morphological transitions during mating under these experimental conditions.

To investigate whether *lip2* plays a role in the penetration phase during infection, microscopic observations of epidermal peals from infected leaves were performed (see Materials and Methods). The leaves were infected with mating cultures of the wild-type or the *lip2* mutant strains of opposite mating type. Five days after infection, filaments produced from the wild-type cross could be observed penetrating plant surface and colonizing host tissue (data not shown). A mixture of yeast-like cells was also found on the plant surface and some of these produced conjugation tubes. There was no defect in the ability to penetrate the leaf surface observed in the leaves infected with the *lip2* mutant strains. Overall, the mutant cells were found in similar stages of development as the wild-type cells: penetrating filaments, yeast-like cells with conjugation tubes and some yeast-like cell on the plant surface. These results suggest that *lip2* is not required for penetration. Taken together, I can conclude (at least from this type of analysis) that Lip2 does not play a role in the early stages of infection.

5.2.6. **Deletion of *lip2* results in more severe disease symptom development in maize seedlings**

After successful tissue colonization by *U. maydis*, the first disease symptoms to appear on leaves and stems include chlorosis and anthocyanin pigmentation. Later, tumors are produced and can develop on all above ground plant parts. The fungus proliferates vigorously within the tumor tissue, and the fungal filamentous cells eventually differentiate to produce spores. Tumors vary greatly in size between infected plants. The most prominent tumors are produced at the base of a stem and these eventually cause the death of the plant. Because the *lip2* gene seemed to have no effect on the initial stages of fungal growth during infection, I further investigated whether the *lip2* influences hyphal proliferation and disease symptom development during later stages of infection. Seven-day old maize plants were inoculated with $10^6$ mL$^{-1}$ cells in a mixture of wild-type (*a1b1* and *a2b2*), of wild-type and mutant (*a1b1* and Δlip2 *a2b2*, and Δlip2 *a1b1* and *a2b2*), or mutant strains (Δlip2 *a1b1* and Δlip2 *a2b2*). Plants were scored for
disease symptoms two weeks after inoculation (Table 5.1). The infected plants were scored by judging the severity of specific symptoms and assigning a numerical rating. A disease index was calculated based on these ratings. The number of plants with and without tumors was also scored (Table 5.1).

Surprisingly, the disease index was higher in plants infected with compatible lip2 mutant strains compared with the mixture of wild-type strains (Table 5.1). Interestingly, the disease index was also higher for the plants infected with the mixture of wild-type and mutant strains. The mutants were able to cause development of all the symptoms typical for the wild-type infections in maize seedlings. However, significantly higher numbers of dead plants and plants with large stem tumors were observed for all those infected with the cell mixtures that included lip2 mutant strains. To confirm the development of more severe disease symptoms, I inoculated maize seedlings with a lower number of lip2 mutant cells (10^5 mL^{-1}). The lower inoculum would usually decrease the symptom development in wild-type infections and, therefore, allow a more accurate evaluation of the difference between the wild-type and mutant infections. As expected, the disease indices were also increased when the plants were infected with the lower concentration of a mating mixture containing lip2 mutant strains or a mixture containing a lip2 mutant strain and wild-type strains (Table 5.1). Therefore, it appears that the deletion of lip2 leads to a hypervirulent phenotype, which results in an increased production of large stem tumors, and eventually death of the plants. The increase in plant death could indicate a lack of a precise regulation of the biotrophic development of the fungus (e.g., fungal proliferation or sporulation) such that more severe symptoms developed. That is, the Lip2 protein may be required to regulate fungal development at a specific phase/phases during infection to promote proper disease progression.

5.2.7. Deletion of lip2 enhances teliospore development

In nature, U. maydis preferentially infects developing ears of mature maize plants. Instead of kernels, tumors develop that are filled with proliferating dikaryotic filaments. The filaments eventually differentiate into diploid teliospores. To examine whether lip2 is required for sporulation and teliospore production, two to three-month old maize plants were inoculated by injecting compatible mating mixtures directly into the silk channels of
developing ears. The mating mixtures contained wild-type (a1b1 and a2b2), wild-type and mutant (a1b1 and Δlip2 a2b2 and Δlip2 a1b1 and a2b2), or mutant strains (Δlip2 a1b1 and Δlip2 a2b2). Teliospore development was observed 10 and 14 days after inoculation (Figure 5.5 and data not shown). After 10 days, the wild-type tumors were white/gray in appearance and cross sections of the tumor tissue revealed cells in various stages of development including branched hyphae with lobbed ends, fragmented hyphae with different cellular morphologies and immature teliospores (data not shown). In contrast, the mutant tumors were visibly darker in appearance (i.e., some of the tumors were completely black) and contained teliospores in different stages of maturation with some sections entirely filled with mature melanized teliospores, and some fragmented hyphae (data not shown). After 14 days, most of the wild-type tumors were black and filled with mostly mature melanized teliospores with echinulated surfaces, and the mutant tumors were completely black and dried up with ruptured plant cell membranes; these contained masses of mature teliospores (Figure 5.5A and B). Furthermore, teliospores isolated from plants infected with the lip2 mutant mating culture showed no differences in germination compared to wild-type spores (Figure 5.6). These observations suggest that lip2 deletion enhances spore development. Overall, these results confirm that Lip2 is important during fungal development.

5.3. Discussion

Phospholipases have been shown to influence virulence in a variety of bacterial and fungal pathogens of plants and humans (Nespoulous et al., 1999; Cox et al., 2001; Ghannoum, 2000). Here I show that deletion of lip2 gene encoding a putative PAF-like PLA2 results in more severe symptom development and increased teliospore maturation. The hypervirulent phenotype suggests that Lip2 protein may attenuate fungal proliferation and/or sporulation. This might be important during biotrophic interactions between the fungus and the host to prevent premature development of disease symptoms.

A connection between the PLA2 activity and adenylyl cyclase regulation has been shown in S. cerevisiae, where products of PLA2 activity, in particular fatty acids, stimulate adenylyl cyclase (Resnick and Tomaska, 1994).
Figure 5.5 Teliospore development on floral tissue by wild-type strains and lip2 mutants.

(A) Tumors were collected from infected ears of maize after 14 days of infection with the wild-type \((a1b1 \times a2b2)\) or the lip2 mutant \((\Delta lip2 a1b1 \times \Delta lip2 a2b2)\) cells. The mutant tumors were visibly darker and dried up in appearance with ruptured cell membranes compared to wild-type tumors. (B) Cross sections of the wild-type and lip2 mutant tumor tissue showing mature teliospores. In mutant tumor tissue, only mature melanized teliospores with echinulated surfaces were observed in contrast to wild-type tumors where teliospores were found in different stages of maturation. The teliospores were visualized by differential interference contrast optics (DIC). Scale bar = 10 \(\mu\)m.
Harvested teliospores from the tumor tissue developed on maize seedlings 14 days after inoculation with the wild-type \((a1b1 \times a2b2)\) or the lip2 mutant \((\Delta lip2 a1b1 \times \Delta lip2 a1b1)\) cells were incubated for germination. Germinating teliospores are black with a thick outer layer (i.e., that does not stain with calcofluor) with an extended promycelium from which haploid progeny are generated (i.e., stained with calcofluor) after a 16-hr incubation period. The teliospores were visualized by differential interference contrast optics (DIC, top panel) and by epifluorescence after staining cell walls with calcofluor (bottom panel). Scale bar = 10 μm.
It is possible therefore that Lip2 could regulate adenylyl cyclase activity in *U. maydis*, and thus regulate cAMP levels in cells during infection. In *U. maydis*, high cAMP levels promote budding growth and low cAMP levels promote filamentous growth. This regulation might be important to keep the disease symptoms in check during the biotrophic phase of *U. maydis* life cycle, so that the plant growth stays unaffected and the fungus can complete its life cycle. In addition, a second product of PLA2 activity, lysophospholipid, inhibits glucan synthase activity in *S. cerevisiae* (Ko et al., 1994). Glucan synthase is a membrane bound protein involved in biosynthesis of cell wall glucan (major cell wall component).

PLA2 cleaves off the acyl group in membrane phospholipids and in some specific bioactive phospholipids resulting in mobilization of arachidonic acid and production of oxylipins (signaling lipids). Oxylipins comprise a family of oxygenated fatty acid-derived signaling molecules that have several biological activities in animals, plants, and fungi. Mammalian oxylipins, including the prostaglandins, mediate many immune and inflammation responses in animals. Prostaglandins production by pathogenic microbes is also theorized to play a role in pathogenesis (Noverr et al., 2001, 2002). Tsitsigiannis et al (2005a) proposed that oxylipins produced by *Aspergillus nidulans* may serve as factors that modulate fungal development to contribute to resistance to host defenses. It is possible that PLA2 activity may directly or indirectly contribute to the weak plant defense response observed during *U. maydis* infection. Oxidized lipid-derived molecules have been shown to play significant roles in inducible plant defense responses against microbial pathogens. The synthesis of these oxylipins was hypothesized to be initiated by the phospholipase A2-mediated release of unsaturated fatty acids from membrane lipids, because linoleic and linolenic acids, the precursors of most oxylipins, dominate the sn-2 position in plant phospholipids (Blee, 1998; Dhondt et al., 2000). Perhaps *U. maydis*, by secreting its own PLA2, may mimic production of a plant PLA2 that cleaves off linoleic or linolenic acids from plant cell membranes, and thus may influence the plant defense response. Therefore, the linoleic or linolenic acids normally released from the membrane upon the plant PLA2 activity would not get mobilized and no induction of subsequent signaling pathways that lead to a plant defense response would be initiated. However, this theory is highly speculative and clues to functions and mechanisms of
PLA2 during host infection must be further investigated, perhaps by conducting a study to examine PLA2 activity during infection.

Many lipids and lipid-derived products that are generated by phospholipases acting on phospholipids present in the host are implicated as mediators and second messengers in signal transduction. Therefore, Lip2 could also contribute to production of signaling lipids that provide important signals necessary for developmental changes during *U. maydis* infection.

Further experiments are needed to assess the connection of cAMP signaling and PFA-like PLA2 in *U. maydis* and the role of this PLA2 in establishment of biotrophic interactions with the host (including a possible role in bypassing plant defense response). For example, experiments could be conducted to determine *lip2* expression in strains deleted in components of cAMP/PKA pathway, such as the *ubcl*, *adr1* and *hgl1* mutants. Also generation of mutant strains deleted in both *lip1* and *lip2* will allow a more detailed exploration of the role of these genes in virulence. Complete elimination of PLA2 activity may result in enhanced pathogenicity in other stages during infection, such as mating or penetration phase. Overall, the results presented in this chapter indicate that *U. maydis* PLA2 plays a role in virulence, specifically by attenuating the disease symptom development during infection. This work therefore strengthens the emerging idea that lipid metabolism and signaling are important for biotrophic interactions of fungi with plant hosts.

5.4. Materials and Methods

5.4.1. Media and Growth Conditions

*U. maydis* strains were grown according to standard conditions (Holliday, 1974). Analysis of lipid-induced filamentation was performed by inoculating 1 x 10⁶ cells in minimal medium supplemented with either glucose, corn oil, palmitic acid or oleic acid (added to a concentration of 1%) and growing strains overnight at 30°C.

Mating tests were performed by co-spotting compatible strains on double complete medium containing activated charcoal (1%) as previously described (Holliday, 1974).
5.4.2. DNA Manipulations

All DNA manipulations, such as small-scale plasmid preparations, restriction enzyme digests and Southern blot analysis, were performed according to standard protocols (Sambrook et al., 1989). Plasmid DNA was isolated using the Eppendorf fast plasmid mini kit and genomic DNA was isolated as previously described (Wang et al., 1988). The sequence of lip2 was obtained from the U. maydis genomic sequence at http://mips.gsf.de/genre/proj/ustilago/ (gene number Um01927). The knock out construct, lip2::hygB<sup>R</sup>, was generated by using a PCR overlap strategy (Davidson et al., 2002), and was designed to include 0.6 kb of the 5' upstream region (left arm) and 1 kb of the 3' downstream region (right arm) from the lip2 gene surrounding a hygromycin selectable marker to ensure a full deletion of the coding region. Primers Lip2P1 (5’ CAGTCGCTCTCTCTTCTTTCT 3’) and Lip2P2 (5’ AACTGTGCTTCAATCGCTGCGATGAAATGGGCGAGACGAGA 3’) and primers lip2P5 (5’ TAGCACACGACTCACAATCTGGCGTAGCATCGAGACGAGAACA 3’) and Lip2P6 (5’ AAGGCAGAAGCGGCGAAGA 3’) were used to amplify the left arm and right arm, respectively, from genomic DNA. Primers Lip2P3 (5’ TTCTCGTCTCTGCTACATCAGCGATGAAAGGCGAAGA 3’) and Lip2P4 (5’ TGTTGCTCTCGATGCTACGACGAGATGCGGCTGCTGCT 3’) amplified a 2.7 kb hygromycin resistance marker region from pIC19RHL. The lip2::hygB<sup>R</sup> construct was generated as the 3.9kb overlap PCR product by PCR using the three PCR fragments as templates and the primers Lip2P3n (5’ GCTCTCTCTCTCTCTCTCTCGA 3’) and Lip2P6n (5’ CGAGAAGCGGCGAAGAAGA 3’). The generated construct was ligated into pCR2.1 (TOPO-TA; Invitrogen) and transformed into E. coli DH10B cells (Bethesda Research Laboratories).

5.4.3. Strains

The deletion strains, lip2::hygB<sup>R</sup> a1b1 and lip2::hygB<sup>R</sup> a2b2, were generated by transformation of wild-type strains a2b2 (518) and a1b1 (521) of opposite mating type using biolistic transformation method (Toffaletti et al., 1993). Transformants were selected on double complete medium with 1M sorbitol and 250µg/ml hygromycin B, and purified
by restreaking for single colonies on complete medium plates containing 150μg/ml hygromycin B. Transformants were screened by colony PCR using a \textit{U. maydis}-specific primer outside the construct Lip2L (5' GAGGACGCGTCGAAGT 3') and a \textit{hygB}-specific primer \textit{HYGBL} (5' -ATC AGT TCG GAG ACG CTG-3'). Gene deletion was confirmed by Southern blot. Two independent deletion mutants in each wild-type strain were selected for all subsequent phenotypic analyses to confirm that the deleted gene is responsible for the observed phenotypes. Representative data from analyses of all of the deletion strains is shown in the results section of this chapter.

5.4.4. Sequence Analysis

Gene prediction, protein alignments and sequence analysis were done using the programs BLAST (Altschul et al., 1997), CLUSTAL W (Thompson et al., 1994), and Pfam (Bateman et al., 2002), respectively.

5.4.5. Microscopic Analysis

For cell wall staining, 1μl of a 20 μg mL⁻¹ solution of fluorescent brightener 28 Calcofluor White (F3543, Sigma) was added directly to 5μl of cell culture on a slide. To observe the production of mating filaments, cells were scraped off of mating medium and resuspended in sdH2O with 1 μL of a 20 μg mL⁻¹ fluorescent brightener 28 Calcofluor White (F3543, Sigma). Cells were observed using a Zeiss Axioplan 2 fluorescence microscope with differential interference contrast (DIC) optics or UV fluorescence to observe cells stained with Calcofluor. Images were captured with a DVC camera and processed with Northern Eclipse imaging software and Adobe Photoshop 7.

5.4.6. Virulence Assays

Strains \textit{albl} (521), \textit{a2b2} (518), \textit{lip2::hygB^R albl} and \textit{lip2::hygB^R a2b2} were grown in PDB medium with overnight shaking at 250 rpm at 30°C. Mating cultures generated by crossing the strains in the following combinations were used to infect maize seedlings: 521 (\textit{albl}) X 518 (\textit{a2b2}), 521 (\textit{albl}) X \textit{a2b2 Δlip2::hygB^R}, 518 (\textit{a2b2}) X
albl Δlip2::hygB<sup>R</sup> and albl Δlip2::hygB<sup>R</sup> X a2b2 Δlip2::hygB<sup>R</sup>. For seedling infections, one-week-old maize plants (Golden Bantam) grown in a greenhouse were inoculated by injecting approximately 100 μL of 1X10<sup>6</sup> or 1X10<sup>7</sup> cells mL<sup>-1</sup> of mating cultures per plant. After 14 days, plants were scored for disease symptoms using the following rating scheme: 1 = chlorosis and pigment production; 2 = small leaf tumors; 3 = small stem tumors; 4 = large stem tumors; and 5 = plant death. Approximately 100 plants for each combination of strains were scored for disease symptoms. For mature plant infections, two to three-month-old maize plants were inoculated by injecting approximately 2 mL of 1X10<sup>6</sup> cells mL<sup>-1</sup> of mating cultures into the silk channels of developing cobs. The infections in seedlings and mature plants were repeated three times. The production of teliospores within tumors was examined after 10 and 14 days.

5.4.7. In planta Phenotypic Analysis

Epidermal peals, the thin surface layer of plant leaf tissue, from 5-day old infected leaves were placed on a 30 μL drop of water with 3 μL (20 μg mL<sup>-1</sup>) of Fluorescent brightener 28 calcofluor white (Sigma, F3543) for microscopic observation. Cross sections of tumor tissue collected from infected mature plants at 10 and 14 days post inoculation were generated using a razor blade. The cross sections were placed on a 30 μL drop of water with 3 μL (20 μg mL<sup>-1</sup>) of Fluorescent brightener 28 calcofluor white (Sigma, F3543) for microscopic analysis.

5.4.8. Teliospore Isolation and Germination

Teliospores were isolated from tumors collected 14 days post inoculation (described above). Whole tumors were sterilized in 10% bleach for 30 seconds, washed twice in sterile dH2O, and ground in 20ml of 1.5% CuSO4.5H2O using sterile mortar and pestle. Ground tumor tissue containing teliospores was filtered through cheesecloth and incubated overnight at room temperature for sterilization. Teliospore suspensions were centrifuged, washed twice in sterile dH2O, and 200μl of the teliospore suspension was incubated on PDA plates containing a microscope slide submerged in the agar. The plates were incubated at 30°C for approximately 16 to 18 hrs. The microscopic slide was
cut out from the agar and 1μl of a 20 μg mL⁻¹ fluorescent brightener 28 Calcofluor White (F3543, Sigma) diluted in 5μl of sterile dH2O was placed directly on a coverslip prior to observations. Teliospores were isolated from three independent experiments and germination was observed in three separate experiments.

5.4.9. Accession Numbers

The sequence data for this work can be found in the EMBL Nucleotide Sequence Submission (EMBL), http://www.ebi.ac.uk, and GenBank, National Center for Biotechnology Information (GenBank), http://www.ncbi.nlm.nih.gov, data libraries under accession number XP_758074
### Table 5.1 Pathogenicity of lip2 mutants

<table>
<thead>
<tr>
<th>Cross or strain</th>
<th>Inoculums (No. of cells)</th>
<th>No. of plants producing anthocyanin</th>
<th>No. of plants with tumors</th>
<th>Total no. of plants infected</th>
<th>% of plants with tumors</th>
<th>Disease score $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a_{1b1} \times a_{2b2}$</td>
<td>$10^6$</td>
<td>16</td>
<td>72</td>
<td>88</td>
<td>82</td>
<td>3.7</td>
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<td>71</td>
<td>74</td>
<td>96</td>
<td>4.3</td>
</tr>
<tr>
<td>$X \Delta_{lip2} a_{2b2}$</td>
<td>$10^6$</td>
<td>5</td>
<td>70</td>
<td>75</td>
<td>93</td>
<td>4.3</td>
</tr>
<tr>
<td>$\Delta_{lip2} a_{1b1}$</td>
<td>$10^6$</td>
<td>6</td>
<td>87</td>
<td>93</td>
<td>94</td>
<td>4.4</td>
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<tr>
<td>$X a_{2b2}$</td>
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<td>33</td>
<td>35</td>
<td>68</td>
<td>51</td>
<td>2.1</td>
</tr>
<tr>
<td>$a_{1b1} \times a_{2b2}$</td>
<td>$10^5$</td>
<td>33</td>
<td>35</td>
<td>68</td>
<td>51</td>
<td>2.1</td>
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<td>45</td>
<td>66</td>
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<tr>
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<td>49</td>
<td>64</td>
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<tr>
<td>$\Delta_{lip2} a_{1b}$</td>
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<td>20</td>
<td>46</td>
<td>66</td>
<td>70</td>
<td>3.5</td>
</tr>
<tr>
<td>$X \Delta_{lip2} a_{2b2}$</td>
<td>$10^5$</td>
<td>20</td>
<td>46</td>
<td>66</td>
<td>70</td>
<td>3.5</td>
</tr>
</tbody>
</table>

$^a$ Table shows combined data from three independent experiments using $1 \times 10^6$ cells, and two independent experiments using $1 \times 10^5$ cells. The pattern of the disease symptom development shown in the table is representative of a pattern observed in each independent experiment.

$^b$ The disease score is calculated as the sum of disease symptoms ratings divided by the total number of infected plants scored for symptoms.
5.6. References


Kämper, J., Kahmann, R., Bölker, M., Li-Jun Ma, L., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Müller, O., Perlin, M. H., Wösten, H. A. B.,


6. DISCUSSION

6.1. The role of lipids in plant-fungal interactions

*U. maydis* has emerged as an important model for plant pathogenic basidiomycetous fungi, a large group of pathogens that cause smut and rust diseases of plants. *U. maydis* is also an important model for developing an understanding of various aspects of biotrophic parasitism. In the past, efforts in the research community led to the identification of some of the key players in signaling pathways that regulate mating, morphogenesis and pathogenicity in *U. maydis*. However, not much research has been done to characterize processes occurring once the fungus is in contact with the host plant. To date, little is known about fungal genes that control or are required for development in the plant, and even less is known about host signals that may contribute to pathogen development. Nutritional requirements of the fungus during biotrophic growth, which coincide with infection, are only just now being explored. The complexity of the maize- *Ustilago* relationship and the obligate nature of part of the fungal life cycle have made it difficult to study the factors required for pathogenic interactions directly. Alternative strategies are therefore necessary to investigate *U. maydis* pathogenesis. One of the approaches is to use fungal cells undergoing infection-related morphological changes in culture (e.g., yeast-to-hyphal transition). These studies are based on the assumption that the genes expressed during the morphological changes would also be expressed during infection in plants. In the work described in this thesis, yeast-to-hyphal morphological changes associated with pathogenesis were used to identify and characterize novel signals contributing to morphogenesis in *U. maydis*. Furthermore, the available *U. maydis* genomic sequence allowed for selection and subsequent targeted gene deletion of candidate genes to characterize the role of lipid metabolism and signaling in the pathogenesis of the fungus.

6.2. Lipids as a novel signal in fungal morphogenesis

As described in Chapter 2, part of this work resulted in a characterization of a novel lipid-related signal that influences morphogenesis of *U. maydis*. Specifically, corn
and other plant oils, and fatty acids serve both as carbon sources and as signals to initiate filamentous growth that resembles the morphology of dikaryotic cells necessary for the infection of host tissue. Levels of lipids as low as 4 nM of palmitic acid induced filamentous growth. Such a low concentration represents an insignificant nutrient source but is comparable with bioactive concentrations for other fungal signals including non-peptidyl fungal sex hormones such as antheridiol or trisporic acid (Gooday, 1983) or surface wax extracts (Podila et al., 1993). For example, the wax extract from avocado at approximately 10 nM was sufficient to induce spore germination and appressorium formation in *C. gloeosporioides*, and this process appears to involve cAMP signaling pathway (Kim et al., 2001). I speculate that lipid signals may be transduced at least in part via the Ras/MAPK pathway in *U. maydis* because I found that mutants lacking the components of this pathway did not respond to lipids by filamentous growth. Both the cAMP/PKA and Ras/MAPK pathways are known to regulate morphogenesis and pathogenesis in *U. maydis*. However, the roles of the pathways appear to be antagonistic with respect to filamentous growth (Kruger et al., 1998). The activated Ras/MAPK pathway stimulates production of filaments (Lee and Kronstad, 2002), but high PKA activity is associated with budding growth (Durrenberger et al., 1998; Gold et al., 1994). Interestingly, both pathways were required for the lipid-induced filamentous growth. Perhaps the MAPK pathway determines perception of a signal indicating the presence of a host, and evaluation of the nutritional status is perceived via the cAMP pathway. A similar interconnection of the two pathways occurs in *S. cerevisiae* to control pseudohyphal growth (Lorenz and Heitman, 1997; Mosch et al., 1999; Rupp et al., 1999).

Studies in other fungi on the role of lipids in morphogenesis highlight the possibility that derivatives of fatty acids, perhaps generated by fungal activities, could serve as signaling molecules. For example, reproductive development (i.e., sporogenesis) of filamentous fungi has been shown to be influenced by perception of oxylipins (Calvo et al., 1999; Goodrich-Tanrikulu et al., 1998; Katayama and Marumo, 1978; Nukina et al., 1981; Rai et al., 1967; Tsitsigiannis et al., 2005b). In addition, oxylipins have been shown to play a role in fungal pathogenesis (Deva et al., 2001, 2000; Noverr et al., 2001, 2002). For example, enhanced prostaglandin production during fungal infection in *C. albicans* and *C. neoformans* appears to promote fungal colonization and chronic infection.
in humans. These fungi are able to produce the prostaglandins *de novo* or via conversion of exogenous arachidonic acid (Noverr et al., 2001, 2002). PLA2 is known to cleave off the acyl group in some specific biolipids resulting in mobilization of arachidonic acid and production of signaling lipids such as oxylipins. Deletion of a gene encoding a putative PLA2 (*lip2*) in *U. maydis* (Chapter 5) enhanced disease symptom development and increased spore germination. This suggests that Lip2 may contribute to production of signaling lipids that provide important signals necessary for developmental changes during *U. maydis* infection. In *S. cerevisiae*, products of PLA2 activity (i.e., fatty acids) stimulate adenylyl cyclase (Resnick and Tomaska, 1994). Therefore, one could speculate that Lip2 may help to regulate fungal proliferation by influencing cAMP levels via adenylyl cyclase and that this process might maintain a balance between proliferation and disease symptom induction during the biotrophic phase for *U. maydis*. One could also imagine that *U. maydis* is capable of modifying host lipids to generate signals that support filamentous growth of the fungus during infection. This potential role of PLA2 in generating endogenous lipid signals during the pathogenic growth of *U. maydis* adds another level of complexity to the role of lipid signaling.

6.3. **The role of β-oxidation in the pathogenesis of *U. maydis***

The nutritional sources available to biotrophic fungi during infection may play a specific role in allowing growth and progression of life cycle *in planta* that is otherwise impossible to achieve in culture. One could imagine that several tightly controlled levels of regulation exist for metabolic genes during fungal growth *in planta*, and that this regulation leads to a controlled progression of the fungal life cycle during host invasion. One possibility is that these fungi acquire the competence to regulate their metabolic genes in response to specific signals from the host plants (e.g., plant lipids). The experimental data described in this thesis (Chapter 2) suggested that fatty acids serve as a carbon source for growth and/or as signaling molecules important for morphogenesis. The presence of lipids and absence of glucose were required for filamentous growth in *U. maydis* (Chapter 2; Klose et al., 2004). The glucose suppression in particular suggests that there is a metabolic component for lipid-induced filamentation. If the lipid response
is relevant to growth in planta, then the timing of utilization of different carbon sources may be important, and specialized interfaces may develop between hyphae and the host cells. Certainly these interfaces in the form of haustorial feeding structures are well described in other biotrophic fungal pathogens (Birch et al., 2006; Mendgen and Hahn, 2002). The work in Chapters 3 and 4 of this thesis described a targeted gene deletion approach to generate mutants impaired in their ability to utilize fatty acids as a way to separate the contributions of carbon source utilization from signaling. That is, the work addressed the question whether the fatty acid-induced morphological change was due to a nutritional signal or a developmental signal? Therefore, genes encoding enzymes in peroxisomal and mitochondrial β-oxidation of fatty acids (mfe2 and had1, respectively) were deleted and characterized to assess the role of lipid metabolism in lipid-induced morphological transition and virulence in U. maydis. What emerges from this study is that the β-oxidation enzymes appear to be required for morphological changes induced by specific fatty acids, however they are not essential for lipid utilization during infection. This suggests that lipid utilization in biotrophic fungi may affect various phases of infection such as, for example, perception of the host plant environment or host defense.

It is interesting that U. maydis appears to possess a mitochondrial β-oxidation system because previous work in yeast concluded that this system was not present in fungi (Chapter 4; Klose and Kronstad, 2006). Deletion of the peroxisomal and the mitochondrial β-oxidation genes revealed that both pathways work together to allow metabolism of a range of different fatty acids in U. maydis. Intriguingly, the two β-oxidation systems were required during specific stages of the U. maydis life cycle during infection. Neither β-oxidation system was required for cell fusion and initial formation of the infectious dikaryon that results from mating reactions between compatible haploid sporidia. Thus mating is independent of the β-oxidation functions as defined by mfe2 and had1. The mitochondrial β-oxidation system seems to be important in early stages of infection, which involve teliospore germination, penetration of a plant surface and establishment of infection. Possibly, mitochondrial β-oxidation allows utilization of alternative carbon sources during early infectious stages when available nutrients are limited, and/or allows utilization of intracellular fungal lipids for infection-related morphological development (e.g., penetration). The peroxisomal β-oxidation system
appears to play an important role in the later stages of development, which involve extensive proliferation in infected host tissue and sporulation. Thus, peroxisomal β-oxidation may be important specifically in the degradation of plant lipids to provide energy to allow extensive proliferation. The activity of this catabolic system might also alter the metabolic profile of intracellular fungal lipids to coordinate developmental changes during infection (e.g., sporulation). The individual developmental stages of *U. maydis* are outlined and the requirements for both Mfe2 and Had1 enzymes are presented in the model (Figure 6.1) to summarize potential roles of β-oxidation during *U. maydis* infection.

In summary, three possible roles for β-oxidation systems in *U. maydis* morphogenesis and pathogenesis may be concluded from the research presented in this thesis. One possible role is to provide the fungus with the ability to metabolize alternative carbon sources such as fungal storage lipids during early stages of infection (mitochondrial β-oxidation) and plant lipids to maintain the extensive proliferation during later stages of infection when the host tissue is depleted of nutrients (peroxisomal β-oxidation). A second possible role is that β-oxidation functions alter the metabolic profile of intracellular lipids to coordinate the infection-related morphological changes or to provide substrates for production of signaling lipids that play a role in morphogenesis. The evidence for the hypothesized signaling role is based on the observations that both Had1 and Mfe2 were required for the filamentation response to specific fatty acids depending on the saturation and carbon chain length of the fatty acid. Therefore, these fatty acids may play a specific signaling role either directly or indirectly after processing to form derivatives such as oxylipins. In addition, the β-oxidation function may also have an indirect effect on morphogenesis by altering composition of intracellular fatty acids. A third possible role for β-oxidation in *U. maydis* may be an influence on plant lipid signaling that may indirectly interfere with a defense response to alter penetration and/or filamentous proliferation. Little is known about the defense response of maize to infection by *U. maydis*. Recently, Basse (2005) demonstrated that *U. maydis* is capable of suppressing a defense response in maize. During biotrophic interactions, highly regulated secretory activities are required from both the fungus and the host to form the interface layers that contribute in an unknown way to the maintenance of compatibility.
and to the lack of host defense mechanisms (Hahn and Mendgen, 2001; Moerschacher et al., 1999). Perhaps the β-oxidation genes are regulated in response to specific signals from the host plants and thus contribute to the formation of the interface layers between the fungus and maize and the suppression of defense responses.

In summary, the research presented in this thesis reveals the critical importance of basic metabolic pathways involving lipids in the development of a fungal disease, not only by providing energy for growth but also by providing substrates for production of potential signaling compounds.

6.4. Future perspectives

The work described in this thesis provides a framework to address the role of lipids in plant-fungal interactions. These novel observations may have general implications for understanding fungal biotrophy with respect to nutritional requirements, pathogen perception of the host environment and plant defense. There are many exciting possibilities for future work, such as further investigation of the signaling aspects and the interaction with the host during U. maydis infection, and a list of possible follow-up experiments is presented below.

1) Examination of the regulation of lip2 and possible connections between PLA2 and cAMP signaling

It would be informative to examine the expression of the lip2 gene in mutants deleted for components of the PKA/cAMP signaling pathway known to regulate morphogenesis and pathogenesis. This could potentially reveal a connection between Lip2 and cAMP signaling and give clues to support a possible role of Lip2 in regulating adenylyl cyclase activity and thus cAMP levels during infection. The regulation of adenylyl cyclase by the products of PLA2 activity is suggested based on studies in S. cerevisiae (Resnick and Tomaska, 1994). Using these studies as a guide, one could also examine adenylyl cyclase activity in extracts of wild type and mutant (e.g., lip2 mutants) cells. In addition, lip2 gene expression could be assessed in fungal cells in tumor tissue to determine whether the gene might play a role in infection.
2) The dissection of the *U. maydis* response to lipid signals using a mutant screen

Mutagenesis and complementation of mutations can be applied as a non-biased approach to discover genes that are important for lipid-induced morphological changes. Characterization of mutant strains that are no longer responding to lipids may reveal whether the morphological response to lipids is relevant to fungal development *in planta*, and therefore important in fungal pathogenesis. In addition, this could identify key components required in plant-pathogen interactions. A screen for mutants unable to respond to fatty acids by filamentation has been initiated and a total of 40 mutants in a *a2b2* strain and 54 in a *a1b1* strain were collected. (Klose and Kronstad, unpublished). Based on the differences in the cellular and colony morphology of the yeast-like mutants, they were separated into eight categories; one hyperfilamentous mutant was also found. The mutants are now ready to be transformed with a cosmid library in an attempt to clone the genes that are defective in these mutants by complementation. The follow-up work on the characterization of the complementing gene(s) may lead to the exciting discovery of factors important for biotrophic growth of *U. maydis*, and perhaps reveal some key functions involved in plant-pathogen interactions.

3) Whole genome approaches to identify genes involved in lipid-induced filamentation

To identify genes involved in morphological changes in response to lipids, genomic approach such as transcript profiling may be used. That is, one could use the recently completed genome sequence and tools such as Serial Analysis of Gene Expression (SAGE) and microarray analysis to compare, for example, the transcripts of the wild-type strain grown under filament-inducing conditions in the presence of fatty acids with glucose-grown cells. This comparison would likely reveal genes encoding factors important to initiate and maintain filamentation and metabolic functions for fungal proliferation on the different carbon sources. In addition, the transcripts of fatty acid-induced filamentous cells of the *mfe2* or *had1* mutant strains could be compared with the mutant cells grown in non-inducing fatty acids. This could result in
identification of genes for proteins important for processing or production of signaling lipid molecules that influence morphological changes in *U. maydis*. In addition, such comparisons may also shed additional light on signal transduction pathways and regulatory elements involved in lipid sensing and lipid-induced morphogenesis. Candidate genes could than be tested for their role in morphogenesis and virulence by constructing deletion mutants and testing these for their ability to undergo morphological changes in the presence of various fatty acids, to proliferate *in planta*, and to induce disease symptoms.

4) Genetic and biochemical studies to explore a role of oxylipins in the induction of morphological and developmental responses in *U. maydis*.

The analysis of types of lipids that trigger filamentation in *U. maydis* has not been exhaustive and one could propose testing other compounds (e.g., oxylipins) for activity. Additionally, the targeted mutation of genes required for oxylipin biosynthesis such as lipoxygenases may elucidate the involvement of oxylipins in pathogenesis of *U. maydis*. For example, mutation in such genes in *A. nidulans* influences the transcription of genes governing sporulation and secondary metabolism (Tsitsigiannis and Keller, 2006; Tsitsigiannis et al., 2004a, 2005b). Therefore, lipoxygenase genes could influence fungal development of *U. maydis* during infection. In addition, endogenous fungal oxylipins have been successfully isolated from several fungal genera (Brodowsky et al., 1992; Nakayama et al., 1996; Su and Oliw, 1996). Therefore, such compounds could potentially be isolated from *U. maydis* and tested for their ability to influence morphogenesis. In addition, not only endogenous fungal oxylipins, but also plant-derived oxylipins have been shown to influence sporulation and secondary metabolism in *Aspergillus* spp. (Burow et al., 1997; Calvo et al., 1999). Furthermore, fungal colonization leads to changes in levels in bioactive oxylipins by activating or repressing seed lipoxygenase gene expression (Burow et al., 2000; Tsitsigiannis et al., 2005a). Therefore, plant oxylipins extracted from maize plants could be tested for their ability to induce developmental responses of *U. maydis*. The *in vitro* tests could include the filament production in the presence of oxylipins with an assessment of the ability to produce branched filaments, clamp-like structures, hyphal septa, and the ability to
Sporulate. These studies may reveal a possible role of plant or fungal oxylipins in regulating the morphogenesis and therefore pathogenesis of *U. maydis*.

5) *Examination of the defense response of maize to U. maydis infection and the role of lipid metabolism in this process.*

Assays for plant defense responses such as the deposition of callose upon penetration, the formation of reactive oxygen species (ROS), or the expression of pathogenesis related (PR) genes could be performed in parallel with observations of phenotypic responses in the host to infections with the wild-type and the β-oxidation mutant strains of *U. maydis*. Some biotrophic fungi such as powdery mildew fungi elicit callose deposition and the extracellular generation of reactive oxygen species in host plants (Huckelhoven and Kogel, 2003; Thordal-Christensen et al., 1997). However, rust fungi, which are closely related to *U. maydis*, often cause no detectable plant defense responses to cell wall penetration, and data suggest that this lack of a plant reaction is due to active suppression by the fungus (Heath, 1998; Skalamera et al., 1997). Therefore, these experiments would initially test whether there are detectable plant defense responses to the wild-type strains of *U. maydis*. Then the difference in plant defenses between the infections with wild-type strains and β-oxidation mutants could be determined. Such observations might provide insight into whether β-oxidation contributes to suppression of defense responses during infection. Some defense responses can be visualized using microscopy after specific treatments. For example, callose deposition can be detected on the plant wall as fluorescent tissue when stained with aniline blue. To assess the formation of ROS, 3,3'-diaminobenzidine-tetrahydrochloride (DAB) can be injected into leaves to visualize H₂O₂ or nitroblue tetrazolium (NBT) can be used to detect superoxide (O₂⁻) generation. The expression of maize pathogenicity related gene PR-1 can also be analyzed (e.g., by RT-PCR) to investigate the plant defense response during infections with wild type or mutant strains (e.g., *mfe2* and *had1*). Maize cultivars resistant to *U. maydis* could also be used for comparisons, although very little is known about the mechanisms of resistance. Furthermore, the cuticle of a leaf, which is the mechanical barrier that the plants protect themselves from invasion by pathogens, can be removed to further assess the ability of
the strains to bypass/suppress the plant defense response. Finally, plants also use secondary metabolites to protect themselves against pathogens and there are three main classes of that could be tested for an influence on *U. maydis*: phenolics, nitrogen based compounds and terpenes. Thus, one might consider testing such compounds for their influence on the growth and morphology of wild type strains and the β-oxidation mutants, especially the *had1* mutant, which appears to be defective in penetration of plant tissue and establishment *in planta*.

<table>
<thead>
<tr>
<th>Fungal development</th>
<th>Role for <em>mfe2</em></th>
<th>Role for <em>had1</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination</td>
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<td>yes</td>
</tr>
<tr>
<td>Mating</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
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</tr>
<tr>
<td><em>In planta</em> proliferation</td>
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<td>?</td>
</tr>
<tr>
<td>Sporulation</td>
<td>partial</td>
<td>?</td>
</tr>
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</table>

**Figure 6.1** Potential roles of β-oxidation during *U. maydis* infection.

A summary of requirements for the peroxisomal (Mfe2) and the mitochondrial (Had1) β-oxidation enzymes during individual developmental stages of *U. maydis*.
6.5. References


