CHARACTERIZATION OF FACTORS INVOLVED IN MATING, MORPHOGENESIS AND VIRULENCE IN SMUT FUNGI

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ABSTRACT

Ustilago hordei and *Ustilago maydis* represent a group of fungal pathogens that cause economically important smut diseases on cereals and grasses. To identify factors involved in pathogenesis, the mating-type locus (*MAT*) was characterized in *U. hordei* and a genetic suppression screen was utilized in *U. maydis*. In *Ustilago hordei*, mating and pathogenicity are controlled by the *MAT* locus, which contains two distinct gene complexes, *a* and *b*. In this study, the *a* and *b* regions were tagged with the recognition sequence for the restriction enzyme I-*Sce*I and determined that the distance between the complexes is 500 kb in a *MAT-1* strain and 430 kb in a *MAT-2* strain. Characterization of the organization of the known genes within *a* and *b* provided evidence for non-homology and sequence inversion between *MAT-1* and *MAT-2*. Antibiotic-resistance markers were also used to tag the *a* gene complex in *MAT-1* strains (*phleomycin*) and the *b* gene complex in *MAT-2* strains (*hygromycin*). Crosses were performed with these strains and progeny resistant to both antibiotics were recovered at a very low frequency suggesting that recombination is suppressed within the *MAT* region. Overall, the chromosome homologues carrying the *MAT* locus share features with primitive sex chromosomes, with the added twist that the *MAT* locus also controls pathogenicity.

In many fungi, mating, pathogenicity and the morphological transition between budding and filamentous growth are regulated by conserved signaling mechanisms including the cAMP/protein kinase A (PKA) pathway and at least one MAP kinase pathway. In this study, suppressor mutants that restored budding growth to a constitutively filamentous *U. maydis* mutant with a defect in the gene encoding a catalytic subunit of PKA were identified. Complementation of one suppressor mutant unexpectedly identified the *ras1* gene. Deletion of the *ras1* gene in haploid cells altered cell morphology, eliminated pathogenicity on maize seedlings and revealed a role in the production of aerial hyphae during mating. An activated

ras1 allele was also used to demonstrate that Ras1 promotes pseudohyphal growth via a MAPK cascade. These results reveal an additional level of cross-talk between the cAMP signaling pathway and a MAP kinase pathway influenced by Ras1.

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LIST OF ABBREVIATIONS

Abbreviation	Term	Abbreviation	Term
BAC	Bacterial artificial chromosome	M	Molar
C	Celsius	MAP	Mitogen activated protein
cAMP	Adenosine 3',5'-cyclic monophosphate	MAPK	Mitogen activated protein kinase
CM	Complete media	MAPKK	Mitogen activated protein kinase kinase
CHEF	Clamped homogenous electric field	*MAT*	Mating type locus
CRE	cAMP-responsive element	Mb	Megabase pair
CREB	cAMP-responsive element binding protein	min	Minute
DCM-C	Double complete media with charcoal	µl	Microliter
drp	Double-resistant progeny	ml	Milliliter
EDTA	Ethylenediaminetetraacetic acid	NAT	Nourseothricin
ERK	Extracellular regulated kinase	NO	Nitric oxide
ETOH	Ethanol	PCR	Polymerase chain reaction
g	Gravity	PDA	Potato dextrose agar
GAP	GTPase-activating protein	PDB	Potato dextrose broth
GEF	Guanine nucleotide exchange factor	Phleo	Phleomycin
GPCR	G-protein coupled receptor	PKA	Protein kinase A
G-protein	GTP-binding protein	PRE	Pheromone response element
GST	Glutathione s-transferase	RFLP	Restriction fragment length polymorphism
H_2O	Water	rpm	Revolutions per minute
HCl	Hydrochloric acid	SAM	Sterile alpha motif
hr	Hour	SDS	Sodium dodecyl sulfate
hyg	Hygromycin	sec	Seconds
kb	Kilobase pair	V	Volts

GENETIC NOMENCLATURE

ORGANISM	GENE		PROTEIN
	WILD TYPE	MUTANT	
Cryptococcus neoformans	*UPPER CASE ITALICS*	*lower case italics*	Sentence case
Ustilago maydis	*lower case italics*	*lower case italics with allele designation*	Sentence case
Saccharomyces cerevisiae	*UPPER CASE ITALICS*	*lower case italics*	Sentence case
Schizosaccharomyces pombe	*lower case italics*	*lower case italics*	lower case

PREFACE

The work presented herein is the culmination of research efforts from 1996 to 2002. Below is the list of papers that have been published as a result of this work, and the contributions made by the candidate:

- **Lee, N.**, Bakkeren, G., Wong, K., Sherwood, J.E. and Kronstad, J.W. (1999) The mating-type and pathogenicity locus of the fungus *Ustilago hordei* spans a 500-kb region. *Proc. Natl. Acad. Sci. U S A*, **96**, 15026-31.

The candidate is responsible for the majority of the work in this study, with the exceptions of the construction of strains 364-86, 364-86dt21 and 365-57, and the plasmid used to construct strain 365-57dt51.

- **Lee, N.** and Kronstad, J.W. (2002) The *ras2* gene controls morphogenesis, pheromone response and pathogenicity in the fungal pathogen *Ustilago maydis*. *Eukaryotic Cell,* **1**, 954-966.

The *ras1* gene designation was changed to *ras2* in this manuscript because of the recent isolation of two Ras genes in *U. maydis*.

ACKNOWLEDGEMENTS

This thesis could not have been written and the work described herein could not have been completed without help from many people I have been fortunate enough to have worked and played with during my graduate career. I am incredibly grateful for the opportunities, guidance, freedom and constant support provided to me by Jim Kronstad. I couldn't have hoped for a better supervisor. I am also thankful to Jim for filling the lab with so many interesting and talented colleagues. Much of my success as a graduate student stems from my interaction with Guus Bakkeren, who taught me many of the skills I use as a scientist now. In addition, I feel lucky to have worked with Kathy Wong and to have received all of the help, encouragement and tools she gave to me for the mapping project.

The depth of support I have received from my mom and dad amazes me, and I can't thank them enough for all of it. I am grateful for the companionship, hot meals and countless pep talks, but I can't even begin to express how much I appreciate the thought behind all of these actions. Special thanks must also be given to my incredible friends, in particular Ana, Ruby and Melanie, who are like family to me and have been there from the start. In addition, I will always be indebted to Francis Chin, Fely Chin and Sherman Quan for helping me through the tough times.

This thesis is dedicated to Kevin, who everyday shows me how to face even the most difficult of challenges and overcome them.

CHAPTER 1: Introduction

1.1 Overview

Fungi are ecologically and economically important organisms that contribute to the food supply and act as antibiotic sources, plant symbionts and infectious agents. While some fungi exist by decomposing dead organic material, others are obligate or facultative parasites. Both plants and animals are susceptible to fungal infections and these hosts share all of the common features of eukaryotic cells with fungi. Thus, the field of fungal pathogenesis presents an opportunity to study fungal biology and the interaction between eukaryotic cells from the perspective of both pathogen and host.

The survival, proliferation and adaptation of fungal pathogens such as the smut fungi *Ustilago maydis* and *Ustilago hordei* involves several challenges including nutrient acquisition, host identification, mating and sexual development. A unique feature of these fungi is that they must mate to initiate infection and a host is required to complete the sexual stage of the life cycle. The need to identify appropriate mating partners and differentiate between host and non-host implies that fungi possess mechanisms to facilitate sensory perception and response. The factors that enable fungi to overcome these challenges can be considered virulence or pathogenicity factors, although the definition of these factors can be controversial. In this study, the intricate connection between mating, signaling and pathogenesis in smut fungi was explored with the goal of identifying fungal pathogenicity factors.

The following introductory chapter addresses the mechanisms by which eukaryotic cells sense and respond to environmental stimuli with particular emphasis on GTP-binding protein (G-protein), adenosine 3',5'-cyclic monophosphate (cAMP)- and mitogen-activated protein (MAP) kinase-mediated signaling. In addition, the specific processes of mating, morphogenesis, infection and signal transduction in *U. maydis* and *U. hordei* are reviewed. Lastly, the manner in

which mating and morphogenesis are regulated in three other well characterized fungi (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Cryptococcus neoformans*) are described for reference and comparison.

1.2 Molecular mechanisms of signal transduction

1.2.1 Extracellular signal perception

There are several systems that enable cells to sense a myriad of environmental cues and initiate the appropriate physiological responses. While hydrophobic molecules may diffuse across cell membranes unassisted, extracellular signals can also be detected through mechanisms that include ion channels, cell membrane associated enzymes and G-protein coupled receptors. For example, nitric oxide (NO) and arachidonic acid are produced by NO synthase and phospholipase A2 in primary cells, but then diffuse to neighboring target cells to activate guanylyl cyclase and protein kinases, respectively (see Beck *et al.*, 1999 and Piomelli, 1993 for reviews). Ion channels are classified based on their selectivity and the manner in which they are opened; voltage-gated channels are regulated by membrane potential and ligand-gated channels are activated upon binding of the ligand to its receptor. Enzymes that are associated with cell signaling may either span the entire membrane, such as receptor tyrosine and serine/threonine receptor kinases, or associate with the cytoplasmic side of the cell membrane, such as Ras (Hubbard and Till, 2000; Lowy and Willumsen, 1993). Finally, G-protein coupled receptors (GPCRs) respond to a wide variety of signals and are typified by the presence of seven transmembrane segments (Bockaert and Pin, 1999). Upon perception of the extracellular stimulus, the signal is transmitted to various molecules within the cell that ensure that the appropriate response is taken (Figure 1.1).

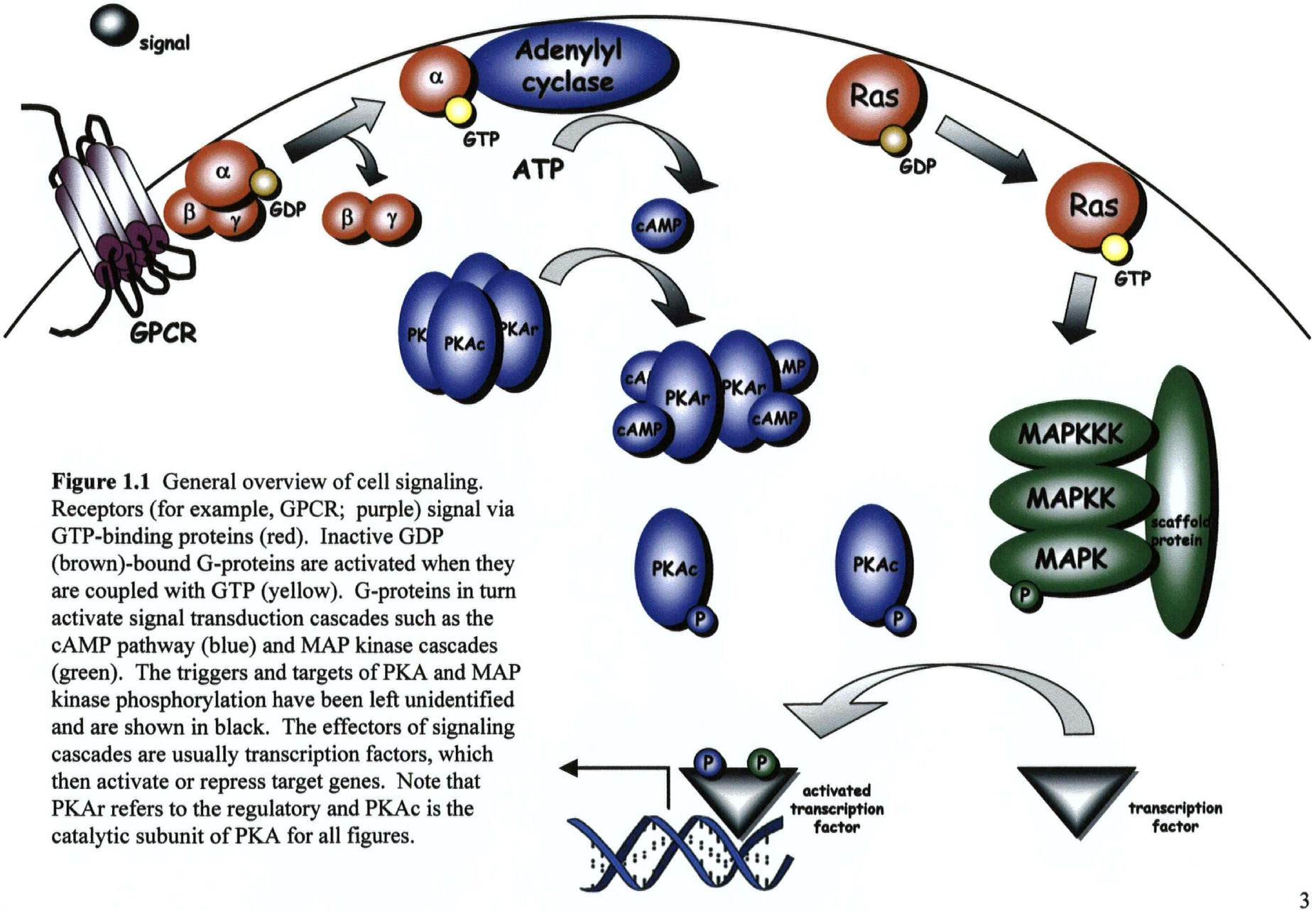


Figure 1.1 General overview of cell signaling. Receptors (for example, GPCR; purple) signal via GTP-binding proteins (red). Inactive GDP (brown)-bound G-proteins are activated when they are coupled with GTP (yellow). G-proteins in turn activate signal transduction cascades such as the cAMP pathway (blue) and MAP kinase cascades (green). The triggers and targets of PKA and MAP kinase phosphorylation have been left unidentified and are shown in black. The effectors of signaling cascades are usually transcription factors, which then activate or repress target genes. Note that PKAr refers to the regulatory and PKAc is the catalytic subunit of PKA for all figures.

1.2.2 GTP-binding proteins

GTP-binding proteins (G-proteins) are found either as monomeric proteins or as heterotrimeric complexes comprised of α, β and γ subunits. GPCRs are usually associated with heterotimeric G-proteins and the binding of agonist to receptor causes the release of GDP from the α subunit. The Gα subunit is then able to bind free GTP, which leads to its dissociation from the Gβγ heterodimer. GTP-bound Gα and/or Gβγ proteins subsequently activate downstream targets until hydrolysis of GTP to GDP causes the reassociation of the α, β and γ subunits (Dohlman and Thorner, 2001; Neer, 1995).

Monomeric G-proteins such as Ras are similar to the Gα subunit of heterotrimeric G-proteins in that they are bound to GTP in their active state and then become inactive upon GTP hydrolysis to GDP (Figure 1.2). Both GTPases appear to use parallel molecular mechanisms and structural elements in GTP hydrolysis. For example, in *S. cerevisiae*, the intrinsic GTPase activity of Ras2p is reduced by specifically mutating glycine 19 to valine (Kataoka *et al.*, 1984; Toda *et al.*, 1985). When comparable mutations are made in Gα proteins, the same impaired GTPase activity is observed (Bourne *et al.*, 1991).

For many GTPases, the intrinsic rates of GDP release and GTP hydrolysis are quite low and may be enhanced by regulatory proteins. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP and promote replacement with GTP. Equally as important are GTPase-activating proteins (GAPs), which aid in GTP hydrolysis (Figure 1.2; Bourne *et al.*, 1991).

1.2.3 Intracellular signaling networks

The targets of G-proteins often serve to amplify the original signals and permit additional levels of regulation. G-proteins activate numerous molecules including adenylyl cyclase, protein

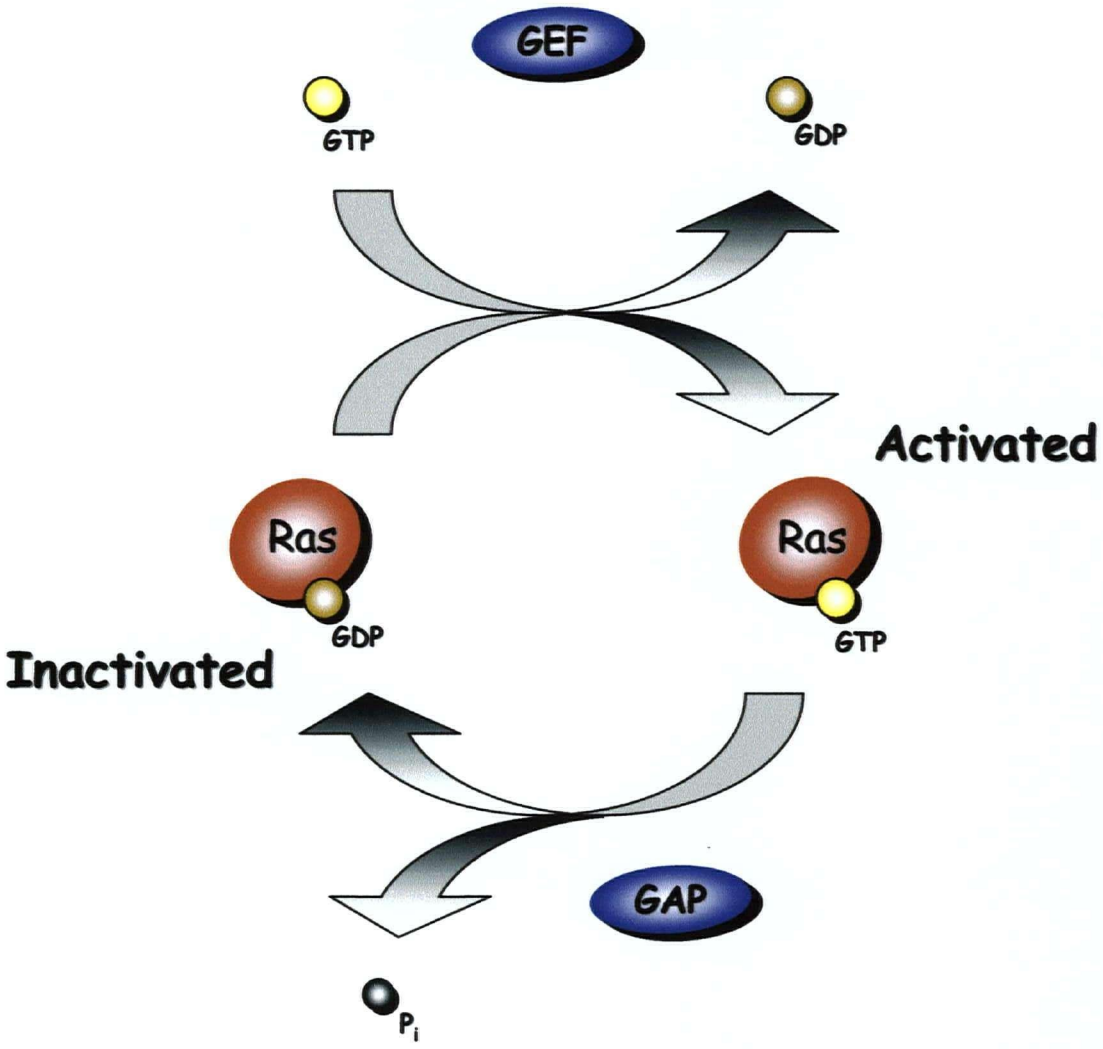


Figure 1.2 The Ras GTPase cycle. Ras proteins (red) are inactive when bound to GDP (brown). Extracellular signals trigger the release of GDP and free Ras to couple to GTP (yellow). GTP-bound Ras proteins are active until GTP is cleaved to GDP, releasing a phosphate molecule (black). Guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) enhance the cycling of guanine nucleotides and are shown in blue.

kinases and phospholipase A2. The cAMP and MAP kinase pathways are highly conserved signal transduction cascades and are discussed in more detail below.

1.2.3.1 The cAMP pathway

Adenylyl cyclase is a membrane bound enzyme that catalyzes the conversion of ATP to the second messenger cAMP, (the first messenger being the extracellular ligand that activates the receptor). The major effector of cAMP signaling is cAMP-dependent protein kinase (protein kinase A or PKA), however, other targets include enzymes involved in glycolysis and G-protein activating GEFs (Bos and Zwartkruis, 1999; Pall, 1981). In its inactive state, PKA is heterotetramer with two regulatory and two catalytic subunits. cAMP activates PKA by binding two molecules for each regulatory subunit and this results in the release of the two catalytic subunits. The catalytic subunits are serine/threonine protein kinases that phosphorylate target proteins containing the PKA phosphorylation site, R(R/K)X(S/T)X. One effector of PKA signaling is the cAMP-responsive element binding protein (CREB), which recognizes the cAMP-responsive element (CRE; TGACGTCA) and induces the expression of genes with regulatory regions containing this consensus sequence (Montminy, 1997).

1.2.3.2 MAP kinase cascades

Mitogen-activated protein (MAP) kinase pathways are activated by a wide variety of signals. MAP kinase cascades are three kinase modules consisting of a MAP kinase, a MAPK kinase and a MAPKK kinase, which are tethered together by a scaffold protein or by direct interaction between the different proteins (Widmann *et al.*, 1999). This organization is thought to permit the use of common components in distinct signaling pathways. The first enzyme that is activated within this module is the MAPKK kinase. This event occurs after interaction with another protein kinase or G-protein and leads to the sequential stimulation of the MAPK kinase

and the MAP kinase. MAP kinases are similar to PKA in that both proteins phosphorylate substrates on serine and threonine residues and the targets are often transcription factors (Treisman, 1996).

The role of signaling cascades in fungal differentiation has recently become an area of intense research. This has led to significant advances in the understanding of how fungi recognize suitable mates, decide on the appropriate response to divergent nutritional supplies and environmental stresses, recognize hosts for infection and overcome host defense mechanisms.

1.3 Mating, morphogenesis and pathogenesis in *Ustilago maydis* and *Ustilago hordei*

1.3.1 The diseases caused by *Ustilago maydis* and *Ustilago hordei*

Ustilago hordei and *Ustilago maydis* represent a group of fungal pathogens that cause economically important smut diseases on cereals and grasses (Figure 1.3; Agrios, 1988; Christensen, 1963; Thomas, 1988). Many smut fungi develop within grain kernels and then eventually replace them with masses of dark teliospores resembling smut or soot. In some cases, the developing spores are surrounded by a membrane that eventually breaks open to release masses of fully developed teliospores. Some of these fungi, such as *U. hordei*, infect germinating seedlings and grow as hyphae within the developing seedlings without causing symptoms. Upon flowering of the host plant, the fungal cells proliferate and then form the teliospores that replace the seeds (Thomas, 1988). Other smuts, such as *U. maydis*, are able to infect all aerial parts of the plant and cause local disease symptoms around the site of infection. With these smuts, spore development takes place within fungal-induced plant tumors that appear to provide the appropriate environment for fungal proliferation and teliospore development (Christensen, 1963). In general, smut fungi have narrow host ranges and only closely related plant species are infected; for example, *U. hordei* causes covered smut of barley and oats,

while *U. maydis* is a pathogen of maize and teosinte (Figure 1.3).

1.3.2 The life cycle of smut fungi

Unlike many fungal phytopathogens, plant infection is possible only during the dikaryotic phase of the smut fungal life cycle. Prior to cell fusion and dikaryon formation, haploid cells are saprophytic and divide by budding. This cell type is easily cultured on artificial media (Holliday, 1974). Initially, two haploid sporidia (1N) exchange peptide pheromones to distinguish self from nonself. Once mating compatibility is established between the two cells, thin, snaking filaments known as conjugation tubes or mating filaments develop from one end of each fungal cell (Martinez-Espinoza *et al.*, 1993; Snetselaar, 1993; Snetselaar and Mims, 1992). Filamentous growth is oriented along a gradient towards the source of the pheromone signal and the conjugation tubes eventually come in contact and fuse (Snetselaar *et al.*, 1996). The emergence of a straight dikaryotic filament from the point of fusion between the conjugation tubes only occurs if the original haploid sporidia are of compatible mating type. Although the nuclei remain separate, the cytoplasm from each progenitor cell fuses (plasmogamy) and migrates into the newly formed infection hypha (N+N). The tip of the dikaryotic hypha grows by apical cell expansion, leaving behind the empty sporidial (parental) cells and cell compartments. The production of infection filaments is easily observed; when compatible haploid cells are co-spotted on mating media, infection hyphae become aerial and give the colony a white, fuzzy appearance (see Figure 4.8). Dikaryon formation signifies the switch from saprophytic to parasitic growth and the host plant environment is absolutely required for sustained growth. Outside of the host environment, the dikaryotic cell type is short-lived and in general, attempts to culture this form have failed (Holliday, 1974; Puhalla, 1968).

For smut fungi such as *U. maydis*, fungal entry into the host may occur via appresoria-like structures (Snetselaar and Mims, 1992; Snetselaar and Mims, 1993), through wounds or through

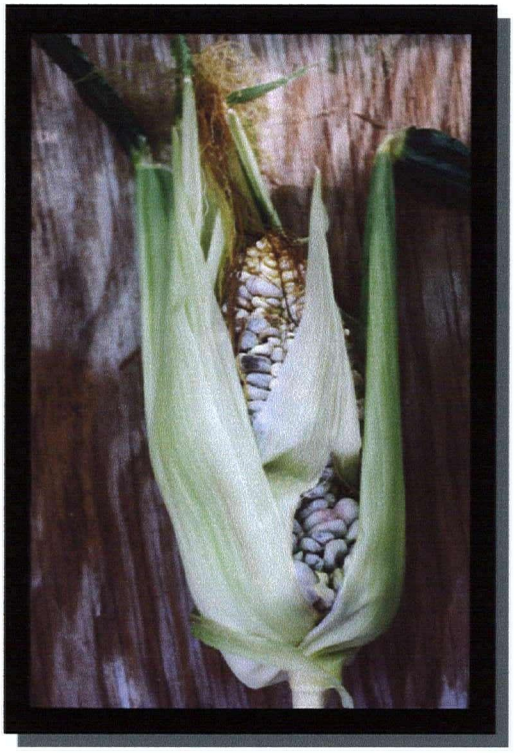


Figure 1.3 The diseases caused by *Ustilago maydis* and *Ustilago hordei*. *U. maydis* is the causal agent of corn smut (left) and infection by *U. hordei* results in covered smut of barley (below). Images obtained from J. Kronstad.

host stomata (Banuett and Herskowitz, 1996). Upon successful host invasion, the fungus proliferates with a filamentous morphology both inter- and intracellularly in host tissue. It is believed that signals derived from the host environment promote filamentous growth *in planta* (Banuett and Herskowitz, 1996). The host responses to fungal infection include stunting, chlorosis, anthocyanin production, tumor formation and plant death. Initially, the plant tumors are composed of enlarged host cells that facilitate fungal proliferation. Over the course of the infection, fungal hyphae fill the tumors and develop branched projections that appear to be mononuclear, indicating that nuclear fusion (karyogamy) occurred (Snetselaar and Mims, 1994). The hyphae then undergo fragmentation and the resulting short fragments and single cells round up to form immature spores. Mature *U. maydis* teliospores (2N) are surrounded by a structured (echinulated) and melanized spore wall that enables spore survival for several years. Fully developed tumors may contain between 2.5 - 6 billion teliospores cm-3 and these spores can be spread by wind and rain to other plants (Christensen, 1963). These spores then germinate under the appropriate nutritional conditions. During spore germination, a short filament (promycelium) protrudes from the spore forming a metabasium in which meiosis takes place. Four haploid nuclei then migrate into individual basidiospore cells (sporidia) that grow with a yeast-like morphology by polar budding. Haploid sporidia are also able to form pigmented, asexual spores (chlamydospores) in response to nutritional deprivation (Kusch and Schauz, 1989).

The life cycle of smuts such as the barley pathogen *U. hordei* is very similar to that described for *U. maydis* above, with the main differences involving interactions with the host. For example, *U. hordei* is a seed-borne fungus that infects germinating seedlings by growing through the coleoptile and into the shoot apex (Thomas, 1988). It is thought that the teliospores become lodged under the hull of the seed and then result in infection of the germinating seedlings. Infected seedlings are occasionally stunted; more commonly, the plants remain

asymptomatic until flowering, when masses of smooth-walled teliospores replace florets within the barley heads (Thomas, 1988). A highly simplified diagram of the general life cycle of smut fungi is illustrated in Figure 1.4.

1.3.3 Regulation of mating, morphogenesis and pathogenesis in *Ustilago maydis* and *Ustilago hordei*

U. maydis and *U. hordei* are classified as heterothallic basidiomycete fungi. Basidiomycetes are distinguished from other organisms within the fungal kingdom by the production of septa within the mycelium and the formation of external basidiospores on the basidium. The term heterothallism refers to the condition of having two or more mating types, with sexual reproduction occurring only when individuals of different types interact. Thus, heterothallic organisms are self-sterile (self-incompatible) and are only capable of mating with compatible mating partners. Compatibility is governed by mating-type loci and ~25% of heterothallic basidiomycete fungi have a single mating-type locus (unifactorial or bipolar system). The remaining 75% of the species have two loci that are responsible for mating (bifactorial or tetrapolar system). For example, *U. hordei* has a bipolar mating system controlled by one mating-type locus (*MAT*) with two alleles or alternative specificities, *MAT-1* and *MAT-2*, while two unlinked loci designated *a* and *b* regulate mating in the tetrapolar smut *U. maydis*. A successful mating interaction is observed only when two cells have different alleles at the *MAT* locus in the case of *U. hordei*, or at both the *a* and *b* loci in *U. maydis*. As mentioned above (Section 1.3.2), sexual compatibility between cells is indicated by the formation of colonies with aerial hyphae (fuz^+ reaction); these combinations are infectious when inoculated into host plants. Conversely, haploid strains or incompatible partners of the same mating type form yeast-like colonies and are non-infectious.

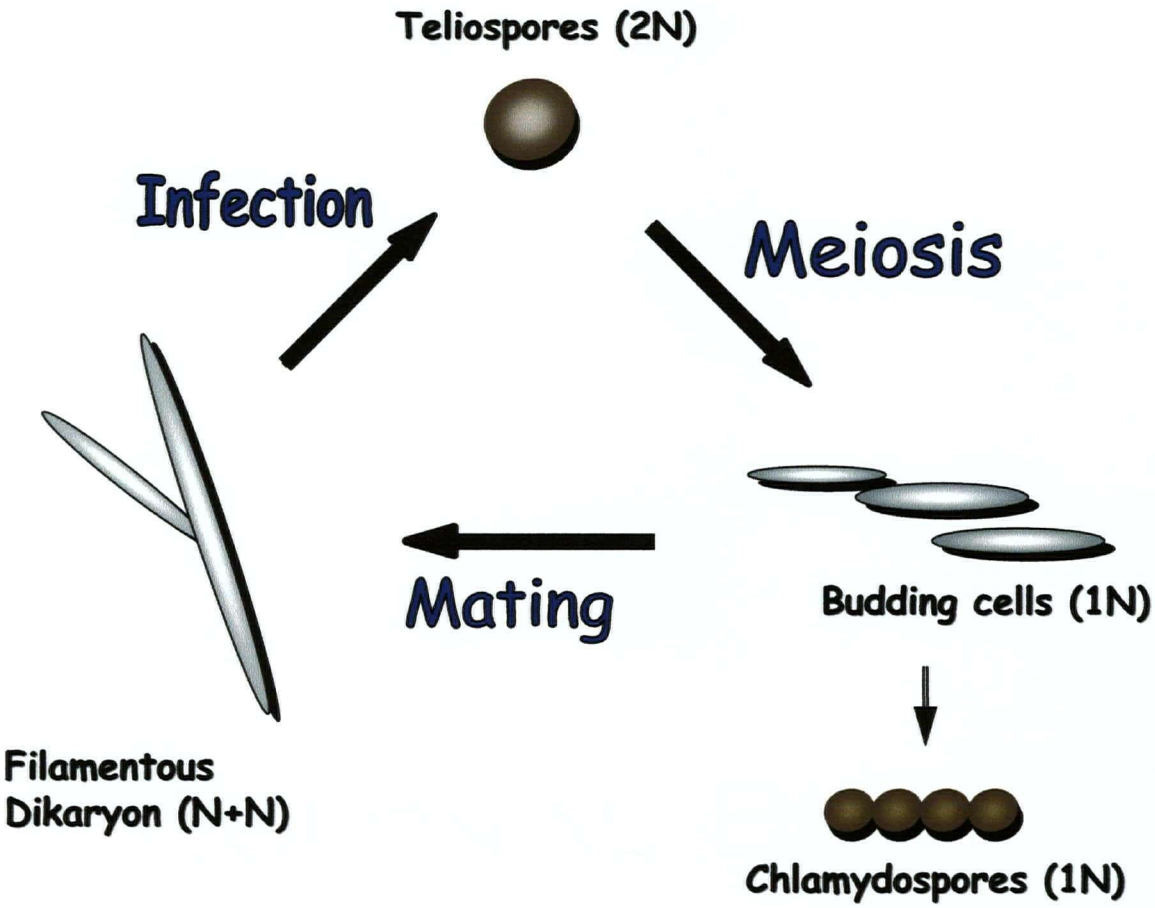


Figure 1.4 The general life cycle of smut fungi. Haploid cells are saprophytic and grow by budding. The dikaryotic cell type is formed after mating occurs and has a filamentous growth morphology. At this stage, smut fungi become infectious and proliferation can only take place within host tissue. Diploid teliospores are produced following karyogamy and then undergo meiosis to form haploid sporidia. A fourth cell type (the chlamydospore) is formed asexually from haploid cells.

In *U. maydis*, the *a* locus is responsible for cell recognition, conjugation tube formation and cell fusion (Banuett and Herskowitz, 1989; Puhalla, 1969; Rowell, 1955; Snetselaar *et al.*, 1996; Spellig *et al.*, 1994a; Trueheart and Herskowitz, 1992). Rowell (1955) used a micromanipulator to isolate and cross individual sporidia from six strains with different combinations of *a* and *b* alleles. Cell fusion was observed in all combinations involving sporidia with different *a* specificities, regardless of the *b* allele; straight-growing, dikaryotic hyphae resulted when the *b* alleles differed and sinuous, slow growing hyphae developed from sporidia homozygous for *b*. Perhaps one of the most convincing experiments demonstrating the function of the *a* locus was performed by Trueheart and Herskowitz (1992). These authors used a cytoduction assay to show that only cells differing at *a* are able to undergo cellular fusion (Trueheart and Herskowitz, 1992). Furthermore, the *a* locus was shown to play a role in intercompatibility between two species that do not normally interact. In this work, a *U. maydis* strain was transformed with sequences from the *a1* gene complex of *U. hordei* and mated with a compatible *U. hordei* strain (*a2*; Bakkeren and Kronstad, 1996).

The *b* locus of *U. maydis* controls filamentous growth, pathogenicity and completion of the life cycle through self vs. non-self recognition between bE and bW polypeptides to establish a regulatory factor (Gillissen *et al.*, 1992; Kamper *et al.*, 1995; Kronstad and Leong, 1989; Schulz *et al.*, 1990). Kronstad and Leong (1989) showed the importance of the *b* locus in pathogenesis by introducing a *b* gene into a haploid strain of opposite specificity at *b* and demonstrating that this strain was sufficient to confer pathogenicity (Kronstad and Leong, 1989). Furthermore, using stable diploids with different specificities at *a* and *b*, Banuett and Herskowitz (1989) were able to determine the specific contribution of the two mating-type loci to filamentous growth and pathogenicity. Diploids differing at *b*, but carrying the same *a* alleles (for example *a1a1b1b2*) had a yeast-like cell morphology on artificial media, but were able to infect maize seedlings and induce tumors. However, diploids heterozygous at *a* and

homozygous at *b* (*a1a2b1b1*) were also yeast-like, but non-pathogenic (Banuett and Herskowitz, 1989). These results show that the *b* locus plays a central role in pathogenicity. To induce filamentous growth on artificial media, the pheromone response pathway must be active because only diploids with different specificities at both *a* and *b*, or diploids homozygous at *a* and heterozygous at *b* (*a1a1b1b2*) supplemented with purified or synthetic pheromone have a filamentous cell morphology (Banuett and Herskowitz, 1989; Bölker *et al.*, 1992; Spellig *et al.*, 1994b).

DNA hybridization experiments with the well-characterized *a* and *b* mating-type genes from *U. maydis* revealed that *U. hordei* possesses similar mating-type functions located at the *a* and *b* gene complexes within the *MAT* locus (Bakkeren and Kronstad, 1993; Bakkeren and Kronstad, 1994). Homologs of the *a* and *b* genes have been characterized in *U. hordei* and demonstrated to be conserved in structure and function compared with the *a* and *b* genes of *U. maydis* (Bakkeren *et al.*, 1992; Bakkeren and Kronstad, 1993; Bakkeren and Kronstad, 1994; Bakkeren and Kronstad, 1996; Martinez-Espinoza *et al.*, 1993). For example, Bakkeren and Kronstad (1996) crossed haploids disrupted for the *b* genes and observed that these mutants are unaffected in their ability to form conjugation tubes that fuse, although they are not pathogenic on barley. Like *U. maydis*, the *a* gene complex controls conjugation tube formation and cell fusion and the *b* gene complex is a central pathogenicity factor in *U. hordei*. Thus, the tetrapolar and bipolar mating systems are distinguished by differences in the genomic organizations of the *a* and *b* genes (Figure 1.5; Bakkeren and Kronstad, 1994).

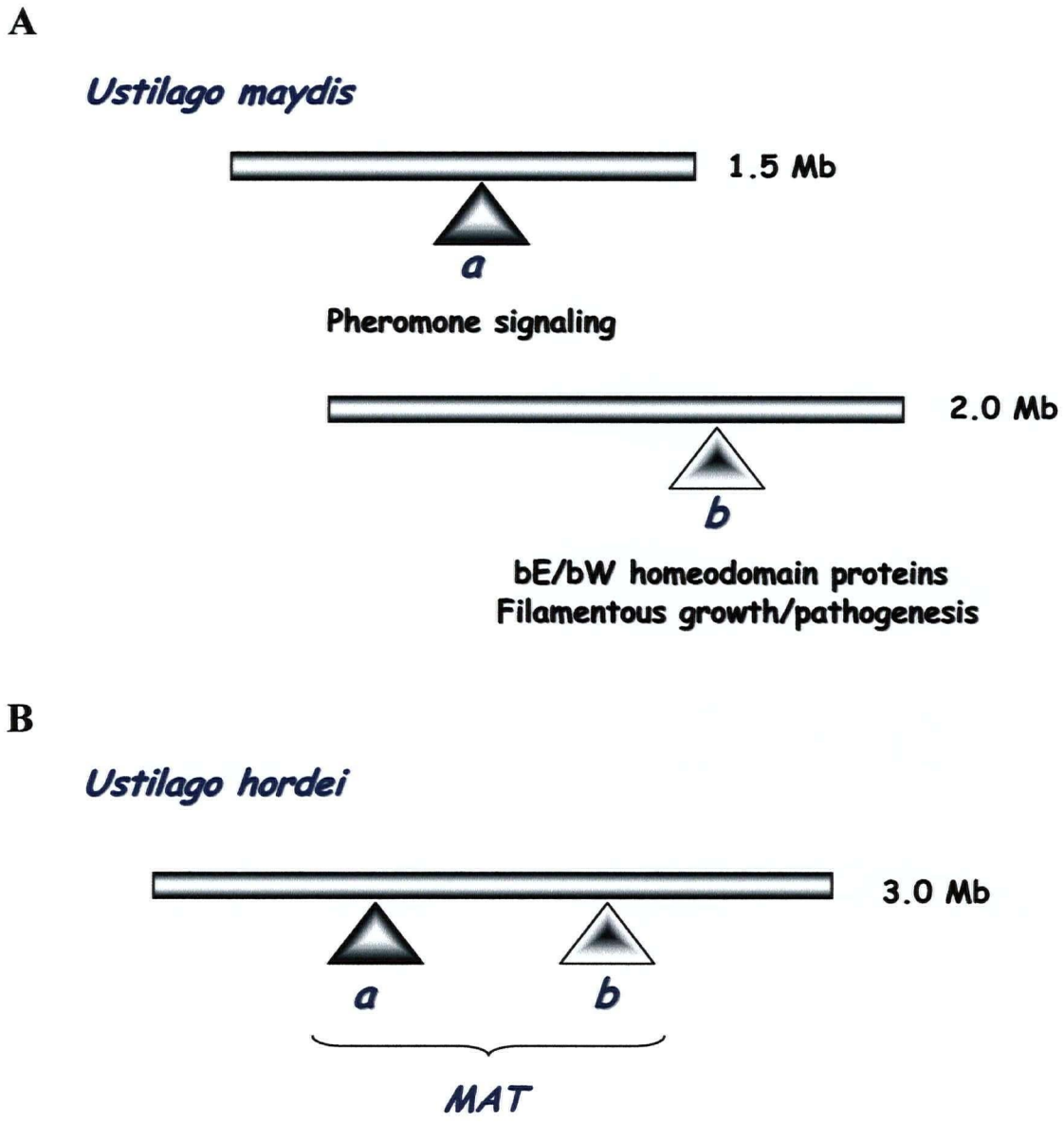


Figure 1.5 Genomic organization of mating-type genes in smut fungi. *U. maydis* has a tetrapolar mating-type system where the *a* and *b* gene complexes are located on separate chromosomes (A). The *a* and *b* gene complexes are found on the same chromosome in the bipolar smut *Ustilago hordei* and define the *MAT* locus (B). The grey bars represent genomic DNA and the approximate sizes of the chromosomes are shown to the right.

1.3.3.1 Molecular requirements for cell recognition and fusion

Over the past decade, a molecular view of the mechanisms behind pheromone response, morphogenesis and pathogenesis in *Ustilago* has emerged to complement the earlier genetic studies described above. The identification and characterization of numerous factors regulating cell-cell communication and pathogenicity have facilitated a deeper understanding of how fungi carry out these events. Among the components that have been described, the pheromone signal and receptor, two signaling modules and the downstream effectors of these pathways play major roles in at least one, if not all three of these processes (Figure 1.6).

1.3.3.1.1 Pheromones trigger conjugation tube formation and cell fusion

In both *U. maydis* and *U. hordei*, two specificities exist for the *a* locus: *a1* and *a2* (Bakkeren *et al.*, 1992; Bölker *et al.*, 1992; Froeliger and Leong, 1991; Rowell and DeVay, 1954). The *a* locus of *U. maydis* was cloned by chromosome walking (Froeliger and Leong, 1991) and by a functional assay for dual mating specificity (Bölker *et al.*, 1992). These sequences at the *a* locus encode cell-type specific lipopeptide pheromone precursors (*mfa*) as well as the putative G-protein coupled, seven transmembrane receptors (*pra*) that recognize pheromones from compatible cells (Bölker *et al.*, 1992; Spellig *et al.*, 1994a). The *U. hordei* genes encoding pheromones and pheromone receptors were cloned by DNA hybridization using sequences from the *a* locus of *U. maydis* and biological assays testing for the formation of mating hyphae (Anderson *et al.*, 1999; Bakkeren and Kronstad, 1994). Sequence comparison between the *pra1* and *pra2* genes encoding pheromone receptors in *U. maydis* and *pra1* and *pra2* from *U. hordei* revealed a respective 62% and 64% nucleotide sequence identity (Anderson *et al.*, 1999; Bakkeren and Kronstad, 1994). At the amino acid level, both the *U. hordei mfa1* and *mfa2* pheromone precursors are 55% identical to their counterparts in *U. maydis*.

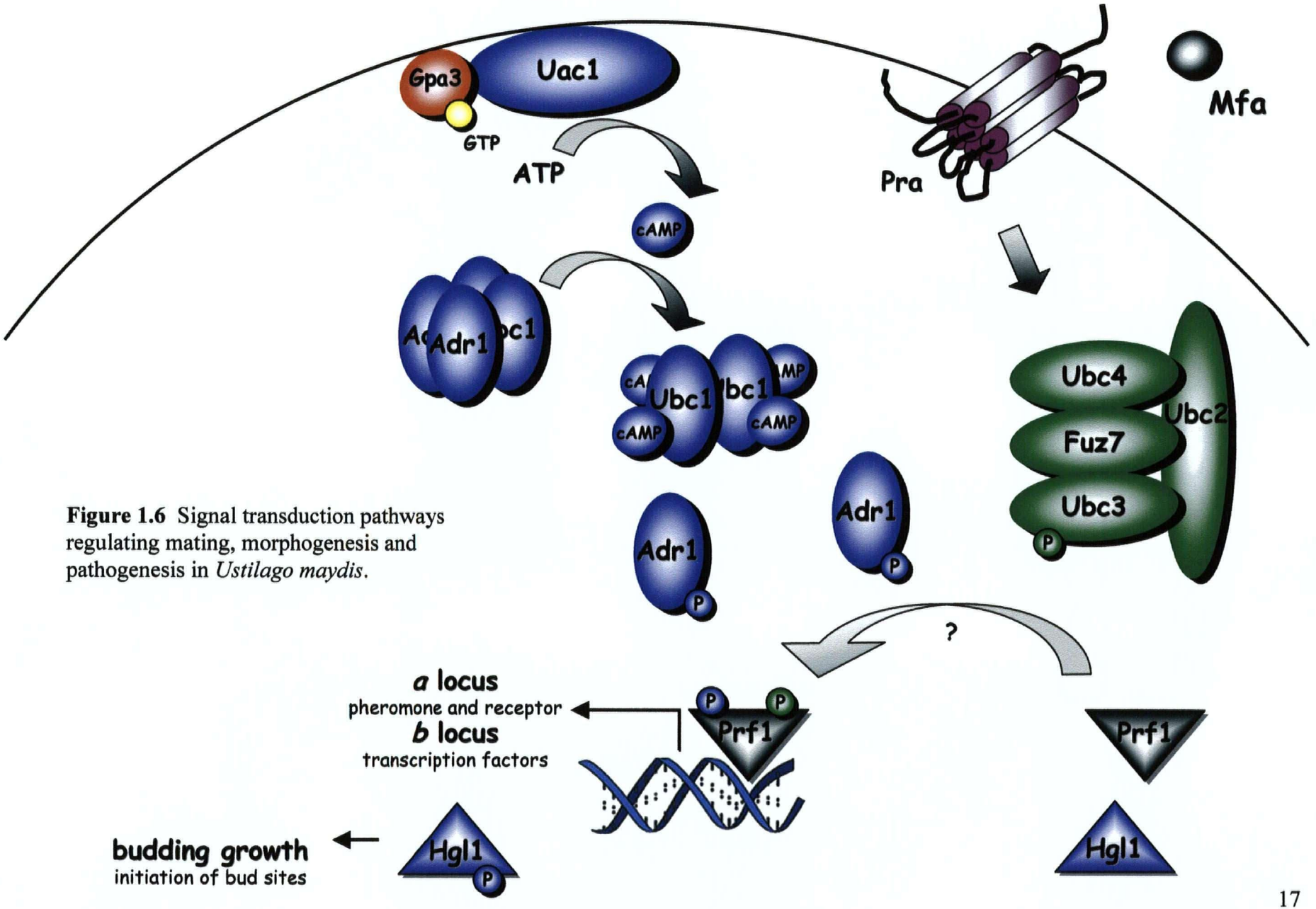


Figure 1.6 Signal transduction pathways regulating mating, morphogenesis and pathogenesis in *Ustilago maydis*.

Pheromones purified from *U. maydis* and *U. hordei* cell suspensions provoke conjugation tube formation in haploids and filamentous growth in diploids (Kosted *et al.*, 2000; Spellig *et al.*, 1994b). These morphological changes are accompanied by a 10- to 50-fold increase in the expression of the *U. maydis mfa1* and *mfa2* genes encoding the pheromones (Urban *et al.*, 1996b). In addition, there is a low (basal) level of pheromone gene expression in wild-type haploid cells and an even lower level of expression in diploid cells heterozygous for both *a* and *b*. The activation of the pheromone response pathway is important for the induction of pheromone gene expression in *U. maydis* because the stimulation of *mfa1* gene expression in response to a2 pheromone secreted from compatible cells is undetectable in haploid *U. maydis* cells deficient for the *pra1* gene (Urban *et al.*, 1996b). Similarly, activation of the pheromone response pathway in both mating partners is necessary for subsequent conjugation tube formation and cell fusion. For example, in *U. hordei*, *MAT-2* (*a2b2*) cells expressing the *pra1* gene (encoding the pheromone receptor from a compatible *MAT-1* strain) are unable to form conjugation tubes and fuse with an engineered tester strain (with the genotype *a2b1*). In constrast, mating hyphae and cell fusion with the tester strain do result when the *mfa1* gene is transformed into *MAT-2* cells (Bakkeren and Kronstad, 1996). These types of experiments provide insight into the roles of the mating-type genes in the regulation of pheromone signaling, cell recognition and cell fusion.

The overall picture of pheromone signaling appears to be that a basal level of pheromone is expressed in haploid cells under the appropriate conditions as a means of attracting compatible mating partners. The recognition of pheromone by pheromone receptors initiates a series of signaling events that leads to the production of more pheromone and pheromone receptors, and the formation of conjugation tubes. The amplification of pheromone signal may be a significant factor in guiding the growth of mating hyphae towards the pheromone-activated cell (Snetselaar

et al., 1996). Finally, upon cell fusion and plasmogamy, the bE-bW heterodimer appears to repress the expression of pheromones and pheromone receptors (Laity *et al.*, 1995).

1.3.3.1.2 A MAP kinase cascade regulates pheromone response

Pheromones trigger the activation of a MAP kinase cascade that is thought to include the MAPKK kinase Ubc4, the MAPK kinase Fuz7, the MAP kinase Ubc3 and the putative adaptor protein Ubc2 (Andrews *et al.*, 2000; Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Mayorga and Gold, 2001; Muller *et al.*, 1999). The *ubc2*, *ubc3*, *ubc4* and *fuz7* genes were all identified using a morphological screen for yeast-like suppressors of a filamentous mutant deficient for the gene encoding adenylyl cyclase (*uac1*; Andrews *et al.*, 2000; Gold and Kronstad, 1994; Mayorga and Gold, 1999; Mayorga and Gold, 2001). The mechanism by which a pheromone induced signal activates this MAP kinase cascade is not clearly understood; however, it is likely that once the signal has reached the MAPKK kinase Ubc4, it is passed to the downstream components Fuz7 and Ubc3. Ubc4 is closely related to the MAPKK kinase Ste11, which controls mating in *S. cerevisiae* (Andrews *et al.*, 2000; Fields *et al.*, 1988).

Using degenerate primers, a homolog of the *Saccharomyces cerevisiae STE7* MAPK kinase was identified in *U. maydis* and called *fuz7* (Banuett and Herskowitz, 1994). Disruption of *fuz7* revealed that this gene is required for full levels of filament formation in haploids during mating and in diploids heterozygous at both *a* and *b*. These results suggest that the *fuz7* gene is involved in both *a*-dependent events (i.e. pheromone response, conjugation tube formation and cell fusion), as well as *a*-independent events leading to the establishment and maintenance of filamentous growth. Andrews et al. (2000) later isolated the *fuz7* gene in an independent study and called the gene *ubc5*. These authors suggest that Fuz7/Ubc5 is a component of a MAP kinase signal transduction cascade regulating pheromone response because they identified *fuz7/ubc5* using the same screen that also yielded other members of the putative mating MAP

kinase cascade and because of the similarity of the morphologies exhibited by double mutant strains mutated at *uac1* and in the genes encoding MAP kinase pathway components (*ubc2*, *ubc3*, *ubc4* and *fuz7/ubc5*). The MAPK kinase encoded by *fuz7/ubc5* will be referred to as the *fuz7* gene in this work.

The *ubc3* gene encodes a putative MAP kinase with 56% amino acid identity to the *S. cerevisiae* MAP kinase encoded by *FUS3* (Mayorga and Gold, 1999). While Mayorga and Gold (1999) identified Ubc3 by complementation, Muller and colleagues (1999) used degenerate primers to isolate a gene encoding a MAP kinase and called this gene *kpp2*. The *ubc3* and *kpp2* genes encode the same MAP kinase and because this locus was first described as *ubc3* in the literature, the gene encoding this MAP kinase will herein be referred to as *ubc3* (Mayorga and Gold, 1998). Deletion of the *ubc3* gene yields mutants that are attenuated for filamentous growth and tumor induction (Mayorga and Gold, 1999; Muller *et al.*, 1999). Specifically, when compared to wild-type strains, compatible *ubc3* mutants co-spotted on mating medium are reduced for filament formation, secrete less pheromone in response to pheromone activation and do not produce conjugation tubes in response to pheromone (Mayorga and Gold, 1999). Furthermore, Ubc3 is involved in both the basal expression of pheromone and the induction of pheromone during pheromone response (Muller *et al.*, 1999).

The *ubc2* gene encodes a putative adaptor protein that may serve to tether the components of the MAP kinase cascade (Mayorga and Gold 2001). Ubc2 contains three domains that are thought to be required for protein-protein interactions: 1) a putative Ras-Association (RA) domain, which indicates a potential interaction with a Ras or other G-protein, (Barnard *et al.*, 1995) 2) a Sterile Alpha Motif (SAM), required for the interaction between the MAPKK kinase Ste11 and the Ste50p of *S. cerevisiae*, (Jansen *et al.*, 2001; Ponting, 1995; Schultz *et al.*, 1997; Wu *et al.*, 1999) and 3) two Src homology 3 (SH3) motifs known to bind proteins with proline rich motifs (Musacchio *et al.*, 1994). Disruption of the *ubc2* gene reduces

filament formation during mating and almost completely abrogates symptom formation during host infection (Mayorga and Gold, 2001). Taken together, these results show that a common theme for MAP kinase pathway mutants exists; mutation of any of the identified components results in faulty pheromone signaling. It should be noted that the pheromone response, MAP kinase pathway in *S. cerevisiae* has guided the thinking in the characterization of the pathway in *U. maydis*.

1.3.3.1.3 The pheromone response factor Prf1 is required for mating, filamentous growth and pathogenicity

Pheromone signaling through the MAP kinase cascade is thought to activate the pheromone response factor encoded by the *prf1* gene (Hartmann *et al.*, 1996; Mayorga and Gold, 1999; Muller *et al.*, 1999). Prf1 has an HMG (high mobility group) box type DNA-binding domain that recognizes and binds to pheromone response elements (PRE; Hartmann *et al.*, 1996). The regulatory regions of all of the genes present at the *a* and *b* mating-type loci contain PREs and two PRE sequences are also found upstream of the *prf1* gene (Hartmann *et al.*, 1996). The activation of Prf1 in response to pheromone explains an observation by Urban *et al.* (1996) that a 10 to 50-fold increase in the expression of the mating-type genes occurs after pheromone stimulation. Mutants deleted for *prf1* are sterile because of an inability to produce and perceive pheromone (Hartmann *et al.*, 1996). One of the main roles of Prf1 seems to be the induction of the mating-type genes because the constitutive expression of the *bE1* and *bW2* genes (see section 1.3.3.2 below) in a solopathogenic strain expressing a *prf1* deletion allele (*a1bW2bE1Δprf1*) restores filamentous growth and pathogenicity to this strain (Hartmann *et al.*, 1996).

1.3.3.2 Molecular requirements for filamentous growth and pathogenicity

The *b* locus of *U. maydis* is multiallelic (Puhalla, 1968; Rowell and DeVay, 1954) and was cloned by transformation of DNA from a *b1* strain into a diploid strain homozygous for the *b2* locus (Kronstad and Leong, 1989). Transformants with the *b2/b2b1* genotype were identified by their filamentous phenotype. Sequence analysis of several alleles revealed two divergently transcribed genes, *bE* (*b*East) and *bW* (*b*West; Gillissen *et al.*, 1992; Kronstad and Leong, 1990; Schulz *et al.*, 1990). The two genes have similar organizations in that each encodes a variable amino-terminal region, a conserved carboxy-terminal region and an intervening homeodomain-related motif (Gillissen *et al.*, 1992; Kronstad and Leong, 1990; Schulz *et al.*, 1990). The *b* locus controls pathogenicity and completion of the life cycle through self vs. non-self recognition by bE and bW polypeptides (Gillissen *et al.*, 1992; Kamper *et al.*, 1995). Using the yeast 2-hybrid system, Kamper *et al.* (1995) showed that bE and bW dimerize only if they are derived from different alleles. The current thought is that self vs. nonself discrimination occurs at the variable amino-terminal ends of bE and bW proteins through hydrophobic effects, polar interactions and/or steric hindrance (Yee and Kronstad, 1993; Yee and Kronstad, 1998).

As mentioned earlier, pheromone signaling induces the expression of the *b* genes (Urban *et al.*, 1996b). However, unlike the genes located at the *a* locus, the expression of bE and bW transcripts remains elevated after cell fusion has occurred (Urban *et al.*, 1996b). Laity *et al.* (1995) also used the cytoduction assay to show that a diploid strain hemizygous at *b* is capable of fusion with a compatible haploid. This is in contrast to the situation in yeast where diploid formation results in repression of mating.

1.3.4 cAMP control of dimorphism and virulence

In addition to the mating-type loci, the cAMP/Protein kinase A pathway regulates the switch from budding to filamentous growth in *U. maydis* (Gold *et al.*, 1994a). In general, high PKA activity leads to a budding phenotype while low PKA activity results in filamentous

growth. This conclusion is based on observations that mutants deficient in the regulatory subunit of PKA (encoded by the *ubc1* gene) display a multiple-budding phenotype, while those lacking the enzyme required to produce cAMP, adenylyl cyclase (*uac1*), or the catalytic subunit of PKA (*adr1*) are constitutively filamentous (Barrett *et al.*, 1993; Dürrenberger *et al.*, 1998; Gold *et al.*, 1994a). In addition to their defects in morphogenesis, mutants deficient in the components of the cAMP pathway are unable to induce tumors and form teliospores *in planta*, demonstrating that PKA signaling also plays an important role in virulence.

In an attempt to identify factors involved in morphogenesis, ultraviolet light was used to mutagenize cells and constitutively filamentous mutants were identified. Transformation of one mutant with a cosmid carrying the gene encoding adenylyl cyclase complemented this defect and restored budding growth (Barrett *et al.*, 1993; Gold and Kronstad, 1994). Despite the correlation between filamentous growth and virulence *in planta*, the filamentous haploid mutant was not found to be pathogenic upon inoculation into susceptible maize plants. Rather, haploid *uac1* mutants are nonpathogenic even after co-inoculation of compatible strains (*a1b2 uac1* X *a2b1 uac1*; Barrett *et al.*, 1993). These results demonstrate that cAMP acts as a key regulator in the switch between budding and filamentous growth. This role is supported by the discovery that exogenous cAMP and mutation of the *ubc1* gene suppresses the filamentous phenotype of *uac1-1* mutants (Gold and Kronstad, 1994). Wild-type and *uac1-1* cells exposed to exogenous cAMP, as well as mutants deficient in *ubc1*, have yeast-like colony morphologies, but exhibit multiple budding cellular phenotypes (Gold and Kronstad, 1994). When assayed for virulence, diploids with homozygous *ubc1-2* mutations and mating mixes of compatible *ubc1-1* mutants are unable to form teliospores, although they are able to proliferate filamentously *in planta* (Dürrenberger *et al.*, 1998; Gold *et al.*, 1994a; Gold *et al.*, 1997; Kruger *et al.*, 2000). Furthermore, crosses between compatible *ubc1-1* mutants resulted in an attenuated filamentous phenotype on complete medium supplemented with activated charcoal (Gold and Kronstad, 1994).

Given the effects of PKA signaling on filamentous growth, Dürrenberger and colleagues (1998) reasoned that defects in the catalytic subunit of PKA would cause constitutive filamentous growth. Two genes encoding PKA catalytic subunits (*uka1* and *adr1*) were cloned by PCR amplification using degenerate primers. Disruption of *uka1* revealed that this subunit plays a minor role in morphogenesis and pathogenesis because *uka1-1* mutants are predominantly yeast-like and able to cause disease in maize. Conversely, *adr1* strains suppress the yeast-like colony morphology of *ubc1-1* mutants and exhibit constitutive filamentous growth, reminiscent of the *uac1-1* mutants. As with the other mutants lacking components of the cAMP pathway, both haploid and diploid *adr1-1* mutants are avirulent *in planta* (Dürrenberger *et al.*, 1998). Taken together, these results show that cAMP signaling is responsible for dimorphic growth, pathogenicity, bud-site selection and cytokinesis. Furthermore, temporal regulation of the cAMP pathway is critical for the completion of the life cycle. Interestingly, the *adr1* gene of *U. maydis* has also been implicated in fungicide resistance (Orth *et al.*, 1995; Ramesh *et al.*, 2001).

1.3.5 Crosstalk between the pheromone response and cAMP pathways

There is mounting evidence implicating the involvement of cAMP signaling in pheromone response. The G-protein α subunit Gpa3 was originally thought to regulate the pheromone response pathway because *gpa3* mutants are unable to induce pheromone gene expression when mixed with compatible strains (Regenfelder *et al.*, 1997). Mutants lacking Gpa3 neither produce infection hyphae nor induce disease symptoms in maize when crossed. It was later discovered that the elongated cellular morphology and mating defect exhibited by *gpa3* mutants are suppressed by the addition of exogenous cAMP (Kruger *et al.*, 1998). The same research group then observed an increase in pheromone gene expression in *ubc1* mutants and wild-type cells grown in the presence of cAMP, compared to wild-type cells grown without

exogenous cAMP. These results led to the placement of Gpa3 upstream of adenylyl cyclase in the cAMP pathway (Kruger *et al.*, 1998).

Further evidence of crosstalk between the cAMP and mating MAP kinase pathways was provided by sequence analysis of the *prf1* transcription factor; putative sites for both MAP kinase and PKA phosphorylation are present in the predicted polypeptide sequence (Hartmann *et al.*, 1996). Strains expressing an altered *prf1* allele (mutated at six putative MAP kinase phosphorylation sites, as well as the putative MAP kinase docking site) are unable to form dikaryotic hyphae when co-spotted on mating media (Muller *et al.*, 1999). In addition, signaling via the cAMP pathway appears to influence pheromone expression via Prf1 on both transcriptional and post-transcriptional levels (Hartmann *et al.*, 1999). Thus, the pheromone response and cAMP pathways may converge on the pheromone response factor Prf1 and influence pheromone signaling in concert (Figure 1.6).

1.3.6 Putative targets of the cAMP pathway in *U. maydis*

As mentioned above, phosphorylation of Prf1 by PKA may represent an additional level of regulation of the pheromone response pathway. Clearly, additional targets must exist that account for the diverse effects produced by perturbations in cAMP signaling. In fact, suppressor analysis of the *adr1* mutant lead to the identification of another putative PKA target, Hgl1 (Dürrenberger *et al.*, 2001). Hgl1 contains nine PKA phosphorylation consensus sites and *in vitro* experiments indicate that Hgl1 may serve as a target for phosphorylation by PKA. Mutants deficient in *hgl1* are able to invade and proliferate within host tissue, but their inefficiency in forming teliospores indicates that Hgl1 may act as a transcriptional regulator of late events in sexual development such as karyogamy, teliospore formation and meiosis. Additional phenotypes of *hgl1* strains include production of a yellow pigment, budding growth alone or in

an *adr1* mutant background, a yeast-like colony morphology and attenuated dikaryon formation (Dürrenberger *et al.*, 2001).

1.3.7 Additional factors regulating dimorphism in *Ustilago maydis*

Pheromones are not the only signals that trigger the switch between budding and filamentous growth in *U. maydis*; several environmental factors also play a role in dimorphism. Kernkamp (1941) experimented with varying concentrations of dextrose in artificial media and discovered that budding growth predominates when dextrose availability is increased. Further studies on environmental influences revealed that filamentous growth is induced by low nutrient availability, exposure to air (presumably O_2 or CO_2) and acidic conditions (Gold *et al.*, 1994a; Kernkamp, 1941; Ruiz-Herrera *et al.*, 1995). Interestingly, recent studies have shown that triacylglycerides and fatty acids also induce filamentous growth in *U. maydis* (J. Klose and J. Kronstad, unpublished observations).

1.4 Mating and morphogenesis in other fungi

The study of similar processes in diverse organisms can reveal conserved mechanisms of regulation and provide valuable clues to identify specialized factors. The sensory response systems for *S. cerevisiae*, *S. pombe* and *C. neoformans* present excellent models for comparison with *Ustilago* and the frameworks for these systems are described below.

1.4.1 Pseudohyphal growth in *Saccharomyces cerevisiae*

The relatively recent rediscovery of the ability of certain *S. cerevisiae* strains to switch from budding to pseudohyphal growth has initiated new interest in using this well characterized organism to identify factors involved in regulating fungal morphogenesis (Gimeno *et al.*, 1992; Kron, 1997). Pseudohyphal growth ensues when these strains are starved for nitrogen and is

thought to enable this non-motile organism to forage for nutrients located at a distance or within natural substrates such as grapes (Blacketer *et al.*, 1995; Dickinson, 1994; Dickinson, 1996; Gimeno *et al.*, 1992). Pseudohyphal growth in diploid *S. cerevisiae* strains is characterized by synchronous, unipolar budding, incomplete cell separation, cell elongation and invasive growth (Gimeno *et al.*, 1992; Kron, 1997; Liu *et al.*, 1996). A similar filamentous form of growth has also been observed in haploid *MATa* and *MATα* cells in response to nutrient limitation (Roberts and Fink, 1994; Wright *et al.*, 1993). However, only haploid cells are capable of invasive growth in rich medium and only diploid cells form pseudohypha that extend beyond the colony perimeter when grown in nitrogen limited medium. The major factors regulating pseudohyphal growth in *S. cerevisiae* are shown in Figure 1.7.

1.4.1.1 Components of the mating MAP kinase cascade regulate filamentous growth

In both diploid and haploid cells, filamentous development is regulated by members of the mating MAP kinase cascade, namely the MAPKK kinase *STE11*, the MAPK kinase *STE7* and the MAP kinase *KSS1* (see Dohlman and Thorner, 2001 for a review). Activation of this MAP kinase cascade by the G-proteins Ras2 and Cdc42 is mediated by a SAM domain containing protein Ste50, the p21-activated protein kinase homolog Ste20 and the 14-3-3 proteins Bmh1 and Bmh2 (Mosch *et al.*, 1996; Roberts *et al.*, 1997). This pathway leads to the derepression of transcriptional regulators Dig1 and Dig2, and subsequent transcriptional activation by a heterodimer composed of Ste12 and Tec1. Genes controlled by a filamentation response element (FRE) including Tec1 itself and the cell surface flocculin Flo11 are then induced by Ste12/Tec1 (Gavrias *et al.*, 1996; Lo and Dranginis, 1998; Madhani and Fink, 1997; Mosch *et al.*, 1996). The *FLO11* gene is one of the few known targets of the pathway and was placed in the filamentation pathway because *flo11* mutants are unable to form pseudohyphal filaments (see below; Lo and Dranginis, 1996; Lo and Dranginis, 1998).

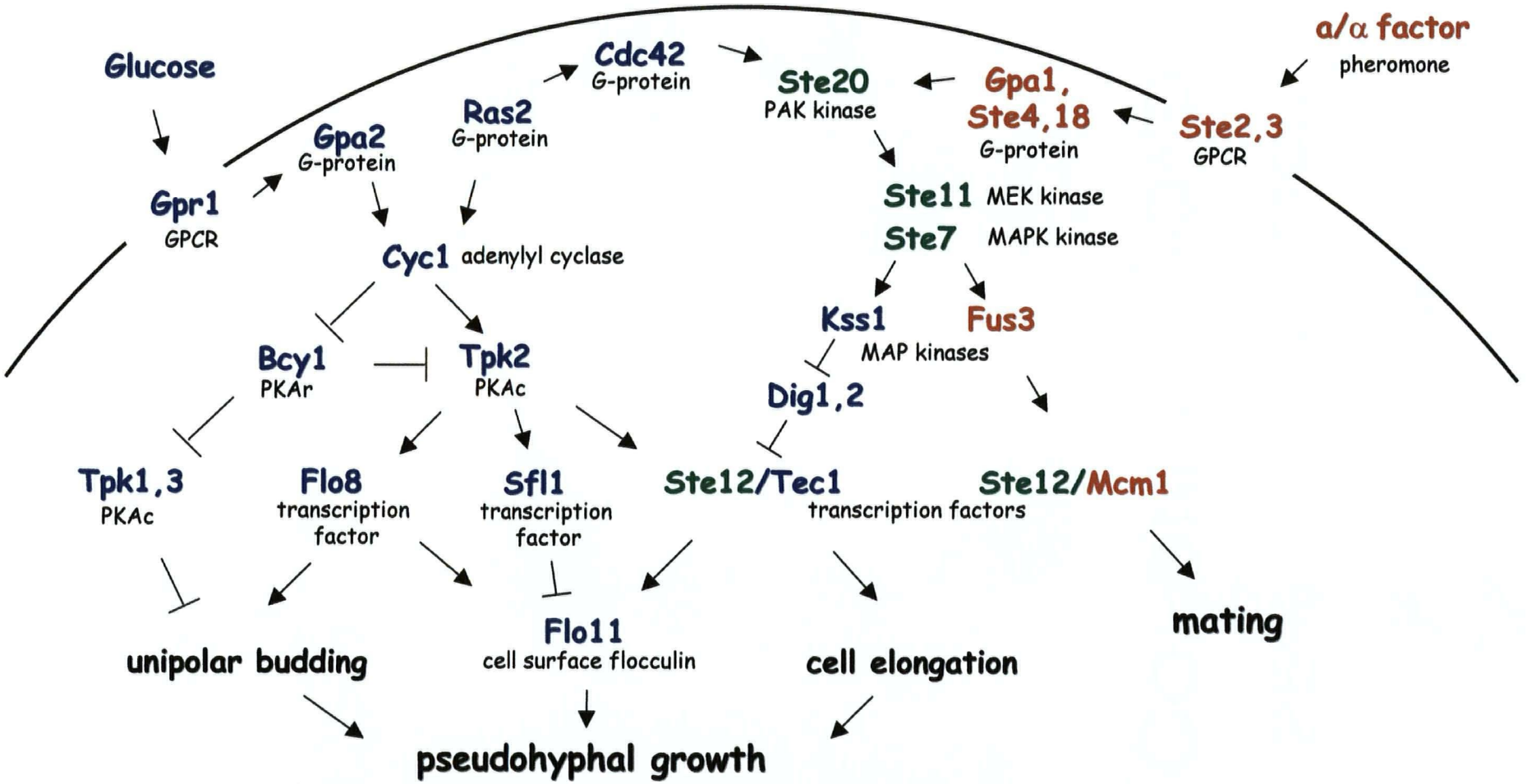


Figure 1.7 Signal transduction pathways regulating mating and pseudohyphal growth in *Saccharomyces cerevisiae*. Proteins involved in pseudohyphal growth are shown in blue, while those required for mating are red. Factors necessary for both processes are in green. Note that on all figures, PKAr refers to the regulatory subunit and PKAc is the catalytic subunit of PKA. Figure redrawn from Lengeler et al. (2000).

1.4.1.2 cAMP signaling controls pseudohyphal differentiation

Signaling by Ras2 results in the activation of not only a MAP kinase cascade, but the cAMP pathway as well (Gimeno *et al.*, 1992; Mosch *et al.*, 1996; Ward *et al.*, 1995). Ras2 acts to elevate cAMP levels by stimulating adenylyl cyclase. A role for cAMP is also indicated because the over-expression of the cAMP phosphodiesterase *PDE2* suppresses the filamentous phenotype of wildtype strains and the enhanced pseudohyphal growth of strains expressing the dominant activated $RAS2^{Val19}$ allele (Ward *et al.*, 1995).

In addition to *RAS2*, a second GTP-binding protein, Gpa2, involved in the modulation of cAMP levels was identified (Nakafuku *et al.*, 1988). This demonstrates yet another level of regulation for the process of pseudohyphal development. The G-protein α homolog *GPA2* appears to act coordinately with *RAS2* to stimulate pseudohyphal differentiation via the cAMP pathway. The addition of exogenous cAMP suppresses the weak defect in pseudohyphal growth displayed in single *gpa2/gpa2* or *ras2/ras2* mutants (Kubler *et al.*, 1997; Lorenz and Heitman, 1997). However, unlike Ras2, Gpa2 does not appear to activate the MAP kinase cascade, but rather also acts on adenylyl cyclase.

Components of the cAMP pathway in *S. cerevisiae* include the G-protein coupled receptor Gpr1, the G-proteins Ras2 and Gpa2, adenylyl cyclase (encoded by the *CYR1* gene), the regulatory subunit of PKA Bcy1 and three catalytic subunits of PKA Tpk1, Tpk2 and Tpk3 (Ansari *et al.*, 1999; Kubler *et al.*, 1997; Lorenz and Heitman, 1997; Pan and Heitman, 1999; Robertson and Fink, 1998; Thevelein and de Winde, 1999; Xue *et al.*, 1998). Gpr1 was identified by its ability to interact with Gpa2 in a yeast 2-hybrid screen (Xue *et al.*, 1998; Yun *et al.*, 1997). Signaling via Gpr1, Gpa2 and the cAMP pathway may be triggered by glucose and ultimately results in the regulation of pseudohyphal development (Ansari *et al.*, 1999; Colombo *et al.*, 1998; Kraakman *et al.*, 1999; Lorenz *et al.*, 2000; Tamaki *et al.*, 2000; Yun *et al.*, 1998).

The three catalytic subunits appear to be redundant for vegetative growth, but play different and opposing roles for pseudohyphal growth; while Tpk2 activates filamentous development, Tpk1 and Tpk3 inhibit this process and may play a role in a negative feedback loop that blocks cAMP production (Nikawa *et al.*, 1987; Pan and Heitman, 1999; Robertson and Fink, 1998).

Several putative PKA targets involved in pseudohyphal development have been identified thus far. These targets include the transcription factors encoded by *SFL1* and *FLO8*; mutants deficient in these genes are either enhanced or defective for pseudohyphal growth, respectively (Kobayashi *et al.*, 1996; Pan and Heitman, 1999; Robertson and Fink, 1998; Tonouchi *et al.*, 1994). Phosphorylation of Sfl1 by Tpk2 inhibits transcriptional repression of *FLO11* via a complex consisting of Sfl1 and the general co-repressor Ssn6-Tup1 (Conlan and Tzamarias, 2001; Keleher *et al.*, 1992; Pan and Heitman, 2002; Robertson and Fink, 1998; Rupp *et al.*, 1999; Smith and Johnson, 2000). Tpk2 also regulates Flo8, which in turn regulates the expression of *FLO11* (Pan and Heitman, 1999; Pan and Heitman, 2002).

1.4.1.3 Crosstalk between the MAP kinase and cAMP pathways

Several examples of cross-talk between the MAP kinase and cAMP signaling pathways exist for the regulation of pseudohyphal growth in *S. cerevisiae*. Firstly, the expression of the cell surface flocculin *FLO11* is regulated by the MAP kinase cascade target Ste12/Tec1, as well as the cAMP pathway targets Sfl1 and Flo8 (Conlan and Tzamarias, 2001; Lo and Dranginis, 1998; Pan and Heitman, 1999; Pan and Heitman, 2002; Robertson and Fink, 1998; Rupp *et al.*, 1999). In addition, the G-protein Ras2 activates both the MAP kinase and cAMP pathways (Mosch *et al.*, 1999; Mosch *et al.*, 1996). Furthermore, an increase in exogenous cAMP levels results in a decrease in the expression of a reporter gene containing the MAP kinase controlled FRE element (Lorenz and Heitman, 1997). Although GST-Ste20 associates with Bmh1 and Bmh2 *in vitro*, Bmh1 and Bmh2 may also be involved in signal transduction by the *RAS2*/cAMP

pathway. Double mutants deficient in *BMH1* and *BMH2* are phenotypically similar to mutants activated in PKA and the expression of $RAS2^{Val19}$ and overexpression of a catalytic subunit of PKA (*TPK1*) suppress glycogen hyperaccumulation by *bmh1bmh2* strains (Roberts *et al.*, 1997). Lastly, Ste12 contains PKA sites and may serve as a target for regulation by cAMP signaling (Lorenz and Heitman, 1998; Mosch *et al.*, 1999).

1.4.2 Signal transduction in *Schizosaccharomyces pombe*

The signal transduction pathways governing sexual development in the homothallic fission yeast *S. pombe* more closely resemble those regulating pheromone response and morphogenesis in *U. maydis* than those controlling mating in the budding yeast *S. cerevisiae*. In *S. pombe*, the combination of glucose limitation, nitrogen starvation and pheromone is required to trigger sexual development. Given the complex nutritional and chemical requirements for mating, it is not surprising that at least two different signaling pathways coordinately regulate this process; a mating MAP kinase cascade and the cAMP signal transduction pathway (Figure 1.8).

1.4.2.1 A MAP kinase cascade regulates pheromone response

Many components of the pheromone response MAP kinase signal transduction pathway have been identified and characterized in *S. pombe*. The P- and M- pheromones and their receptors, map3 and mam2, are required not only for early mating events such as cell recognition and fusion, but also for later events during the life cycle such as meiosis and sporulation (Imai and Yamamoto, 1994; Kitamura and Shimoda, 1991; Kjaerulff *et al.*, 1994; Tanaka *et al.*, 1993; Willer *et al.*, 1995). It is likely that each pheromone-specific receptor is coupled to the α subunit of a heterotrimeric G protein because *gpa1* mutants are unable to respond to pheromone (Obara *et al.*, 1991). Gpa1 then acts in concert with another G-protein encoded by the *ras1* gene to

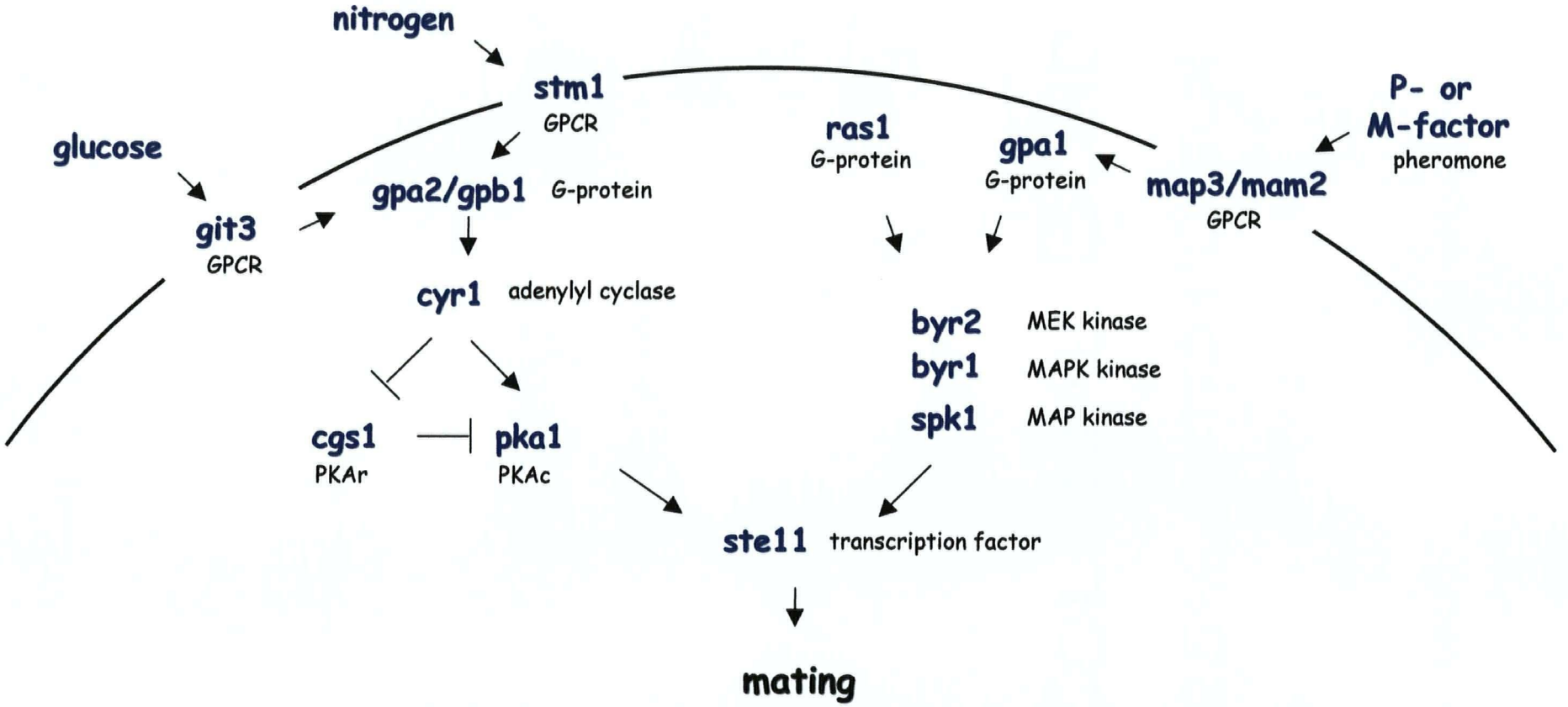


Figure 1.8 Signal transduction pathways regulating mating in *Schizosaccharomyces pombe*.

regulate pheromone response (Xu *et al.*, 1994). Deletion of *ras1* results in cells that are shorter and rounder than wild-type cells and that fail to respond to pheromone (Fukui and Kaziro, 1985; Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986). The target for ras1 is the MAPKK kinase byr2, which becomes translocated to the plasma membrane upon activation of ras1 (Bauman *et al.*, 1998; Masuda *et al.*, 1995). Activation of byr2 is facilitated by the *S. cerevisiae* Ste20 homolog shk1 and results in the sequential activation of the MAPK kinase byr1 and the MAP kinase spk1 (Marcus *et al.*, 1995; Nadin-Davis and Nasim, 1988; Toda *et al.*, 1991; Tu *et al.*, 1997). The transcription factor ste11 contains two potential MAP kinase phosphorylation sites and is required for pheromone-dependent gene expression (Aono *et al.*, 1994; Kjaerulff *et al.*, 1997; Petersen *et al.*, 1995; Sugimoto *et al.*, 1991). The ste11-binding site (TR-box) is present within the regulatory regions of every pheromone induced gene characterized thus far. Furthermore, *ste11* mutants are sterile, while strains over-expressing *ste11* undergo sexual development regardless of nutritional conditions (Sugimoto *et al.*, 1991).

1.4.2.2 The cAMP pathway controls sexual development in response to nutrients

Sexual activity is repressed during mitotic growth and it is only after nutrients become limiting that *S. pombe* cells cease to grow and instead shift to mating. While glucose starvation causes a sharp and rapid decrease in cAMP levels, nitrogen limitation results in a gradual and moderate decrease (Maeda *et al.*, 1990; Mochizuki and Yamamoto, 1992). This reduction in cAMP signaling is thought to trigger sexual development, as mating is inhibited when exogenous cAMP is added to nutrient-depleted media (Calleja *et al.*, 1980). Two putative seven-transmembrane G-protein coupled receptors have been identified, stm1 and git3. In response to nitrogen starvation signals, *stm1* is transcriptionally induced and the stm1 protein product interacts with a Gα protein gpa2 (Chung *et al.*, 2001). The git3 gene is homologous to Gpr1 from *S. cerevisiae* and thought to serve as a glucose receptor (Welton and Hoffman, 2000). The

phenotype of git3 mutants is identical to strains deficient in gpa2, Gβ protein (gpb1), adenylyl cyclase (git2) and PKA (pka1) in that sexual development is derepressed during mitotic growth on rich medium (Hoffman and Winston, 1990; Isshiki *et al.*, 1992; Kim *et al.*, 1996; Maeda *et al.*, 1990; Maeda *et al.*, 1994; Welton and Hoffman, 2000). Conversely, mutants deficient in the regulatory subunit of PKA, (encoded by *cgs1*), are inhibited for sexual development (DeVoti *et al.*, 1991). Interestingly, these results imply that a Gβ subunit works in conjunction with a Gα subunit to activate cAMP signaling in response to glucose-activated git3. One of the targets of PKA phosphorylation is the transcription factor ste11 (Sugimoto *et al.*, 1991). The exact mechanism behind cAMP inhibition of *ste11* expression is not known, however, it is clear that the regulation of ste11 activity is quite complex because signals stemming from the stress-induced MAP kinase cascade and the mating MAP kinase pathway also converge on this target (Shiozaki and Russell, 1996; Sugimoto *et al.*, 1991). This situation appears to be similar to the regulation of the Prf1 transcription factor in *U. maydis* (Figure 1.6).

1.4.3 Mating and signal transduction in *Cryptococcus neoformans*

C. neoformans is a human pathogen and the leading cause of fungal meningoencephalitis. Despite differences in host specificity, *U. maydis*, *U. hordei* and *C. neoformans* share many similarities including the fact that all three organisms are heterothallic, basidiomycete fungi.

1.4.3.1 *The C. neoformans* mating-type locus

C. neoformans has a bipolar mating-type system and the two mating-type specificities are known as MATa and MATα. Among the strains collected from natural and clinical settings, the MATα mating type appears to be 30-40 times more prevalent (Kwon-Chung and Bennett, 1978). Only strains with the MATα mating type are capable of haploid fruiting, a phenomenon whereby

nitrogen starvation induces haploid cells to undergo filamentous growth and sporulation in the absence of a mating partner (Erke, 1976; Wickes *et al.*, 1996). Furthermore, MATα strains are more virulent than MATa strains in a murine model of cryptococcosis (Kwon-Chung *et al.*, 1992). These findings have prompted an investigation of the sequences at the MATα mating-type locus. Recent investigations of the α mating-type locus have identified a 50-kb α-specific region that contains multiple genes involved in pheromone response including the *S. cerevisiae* *STE12*, *STE11* and *STE20* homologs, as well as three copies of the mating-type α pheromone gene and a pheromone receptor (Figure 1.9A; Karos *et al.*, 2000).

1.4.3.2 Ras1 signals through cAMP and MAP kinase pathways to control mating, filamentation and virulence

Two Ras genes have been identified and characterized in *C. neoformans* (Alspaugh *et al.*, 2000; Waugh *et al.*, 2002). Mutants deficient in *RAS1* are unable to mate, adhere to agar, grow at 37°C and maintain full infection in a rabbit model of cryptococcosis. Although the *ras2* mutation has no discernable effect on growth, differentiation and virulence, overexpression of *RAS2* fully restores mating and partially suppresses the high temperature growth defect in a *ras1* mutant. Evidence suggests that Ras1 activates both a mating MAP kinase cascade whose components include the G protein β subunit Gpb1, p21-activated protein kinase homologs (Ste20a, Ste20α and Pak1), the MAP kinase homolog Cpk1 and the transcription factor Ste12, as well as the cAMP pathway, composed of the Gα protein Gpa1, adenylyl cyclase (Cac1) and the regulatory (Pkr1) and catalytic (Pka1) subunits of PKA (Alspaugh *et al.*, 2000; Alspaugh *et al.*, 1997; Alspaugh *et al.*, 2002; D'Souza *et al.*, 2001; Wang *et al.*, 2002; Wang *et al.*, 2000; Waugh *et al.*, 2002; Yue *et al.*, 1999). For example, expression of the activated $RAS1^{Q67L}$ allele in *ste12α* mutants fails to induce filamentous growth and overexpression of *GPB1* suppresses the

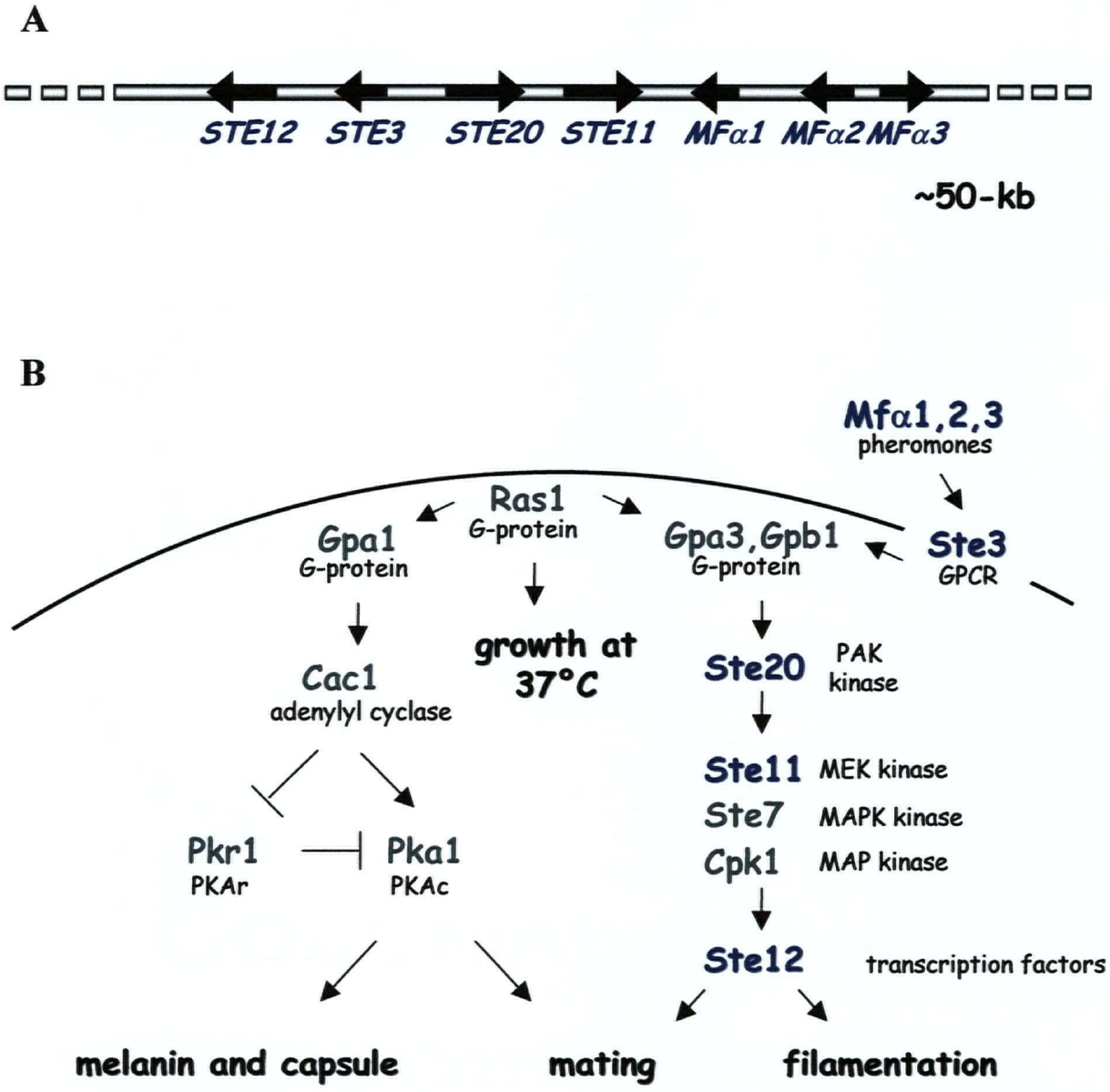


Figure 1.9 Physical and genetic loci involved in mating, filamentation and virulence in *Cryptococcus neoformans*. Genomic organization for part of the MATα locus (A). The grey line represents genomic DNA and the black boxed arrows denote the direction of transcription of the genes. Note that only mating specific genes are shown here. Adapted from Karos et al. 2000. Signal transduction pathways regulating mating, filamentation and virulence (B). Proteins highlighted in blue are represented in the MATα locus shown in (A).

mating defect of *ras1* strains showing that Ras1 acts through the MAP kinase pathway to regulate mating and haploid fruiting (Alspaugh *et al.*, 2000). Furthermore, the addition of cAMP partially restores the ability of *ras1* mutants to mate. However, the *ras1* high temperature growth defect is unaltered by enhancing cAMP and/or MAP kinase signaling and *RAS1* has no effect on PKA specific phenotypes such as melanin and capsule production. Thus, the Ras1, cAMP and possibly MAP kinase signaling pathways regulate overlapping as well as unique functions (Figure 1.9B).

1.5 Mating-type in other fungi

Mating is an important process in the life cycle of sexually reproducing organisms and leads to genetic variability within the population. In fungi, mating-type (*MAT*) loci serve to distinguish self from non-self and regulate development. Mating-type loci are composed of a cluster of two or more genes that govern mating and at least three different structures have been described for fungal mating-type loci. The first involves three different copies of the *MAT* locus, with only one being active at any given time. This type of system has been described for the pseudohomothallic yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Haber, 1998). A second class can be found in approximately 75% of heterothallic (self-sterile) basidiomycete fungi and homothallic (self-fertile) fungi such as *Coprinus cinereus* and *Schizophyllum commune* (Kothe, 1999; Kronstad and Staben, 1997; Kues, 2000). These fungi possess a tetrapolar (bifactorial) mating-type system that is composed of two unlinked loci that encode pheromones and pheromone receptors at one locus and homeodomain proteins that act as transcriptional regulators at the second locus. The third class of mating-type loci is termed bipolar (unifactorial) and is made up of a single locus containing genes required for mating, including pheromones, receptors and transcription factors. All heterothallic ascomycetes and approximately 25% of the heterothallic basidiomycetes have bipolar mating systems (Kronstad

and Staben, 1997). In addition, the mating-type locus of some homothallic fungi may either be non-functional or contain a combination of linked genes or gene fusions permitting self-compatibility (Kronstad and Staben, 1997; Yun *et al.*, 2000; Yun *et al.*, 1999). The *MAT* loci from *S. cerevisiae*, *Coprinus cinereus* and *Schizophyllum commune* are discussed further for comparison with the mating systems in the smut fungi.

1.5.1 Mating-type in *Saccharomyces cerevisiae*

S. cerevisiae exhibits three cell types depending on the genes present at the *MAT* locus: haploid α cells, haploid **a** cells and diploid **a**/α cells (Haber, 1998). There are two alleles for the mating-type locus, *MAT*α and *MAT***a**. The two alleles differ by a region of 700-bp called Yα in *MAT*α cells and Y**a** in *MAT***a** cells (Figure 1.10A). This region contains most of the open reading frames of two divergently transcribed genes that control many mating related activities. The rest of the locus is divided into four more segments called W, X, Z1 and Z2 (Figure 1.10A). The two proteins encoded at the *MAT* locus are called Matα1 and Matα2 in α cells, and Mat**a**1 and Mat**a**2 in **a** cells. In *MAT*α cells, Matα1 acts with Mcm1 to activate α-specific genes encoding proteins such as α factor and the **a** factor receptor Ste2. Furthermore, the genes encoding **a** factor and the receptor for α factor are repressed by the homeodomain protein Matα2, in conjunction with Mcm1, Tup1 and Ssn6. There is no known function for Mat**a**1 and Mat**a**2 in haploid cells, however, in diploids, Mat**a**1 combines with Matα2 to repress the expression of *MAT*α1 and thereby inhibit the expression of α-specific genes. Mat**a**1/Matα2 also act to repress the expression of haploid-specific genes encoding components of the pheromone response pathway (Haber, 1998).

Although there are two different alleles for the *MAT* locus, all yeast cells are basically composed of the same genetic material and have the capacity to express both **a** and α specific

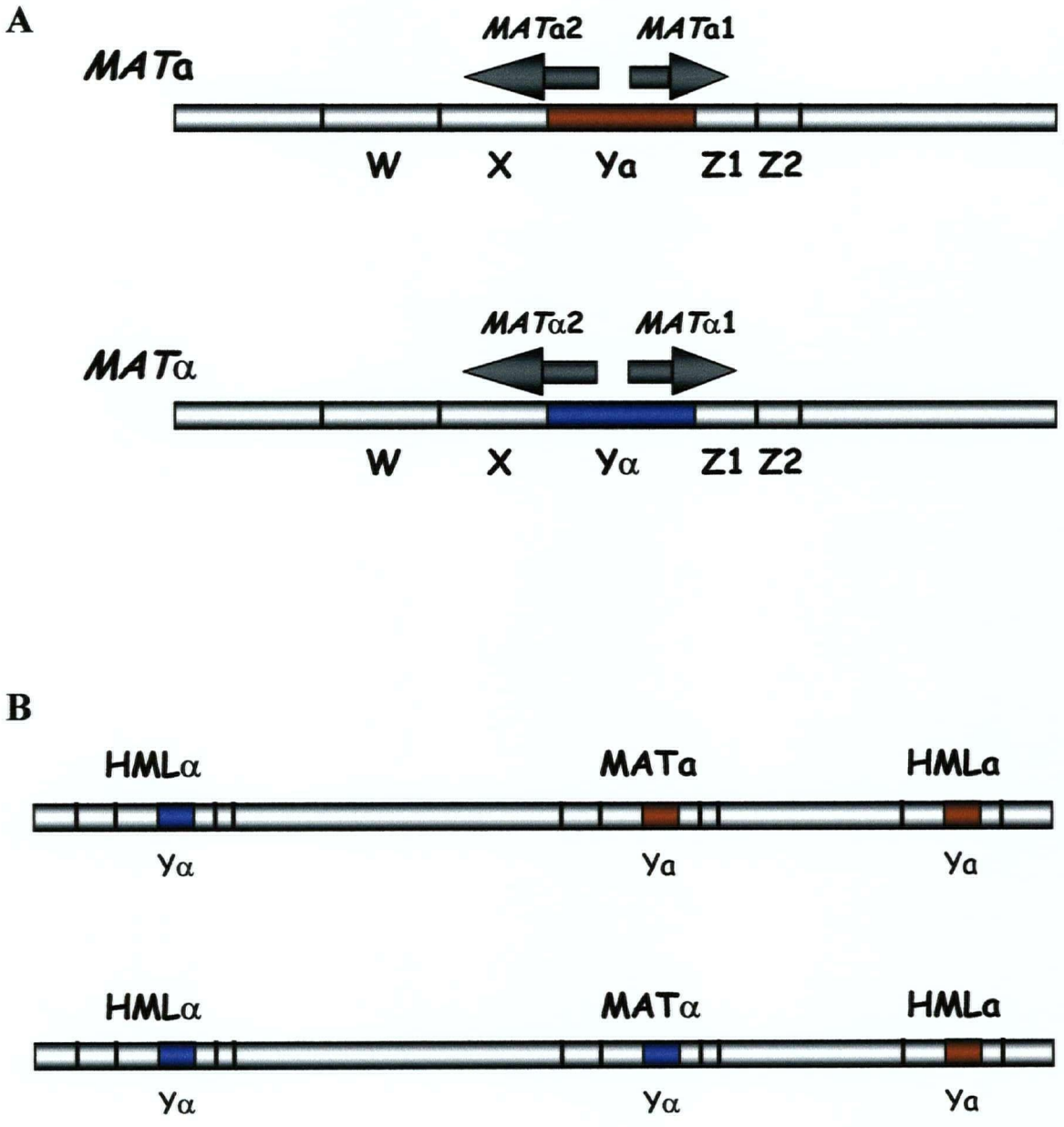


Figure 1.10. Genomic organization of mating-type genes in *Saccharomyces cerevisiae*. The *MAT* locus is divided into five segments and mating type is determined by the presence of either the Y**a** (red) or Yα (blue) cassette (A). The mating-type loci on chromosome III include the MAT locus and the unexpressed HMLα and HMR**a** loci (B). The grey bars represent genomic DNA and the mating-type genes are shown as grey boxed arrows denoting the direction of transcription.

mating-type genes. Two unexpressed copies of the *MAT* locus exist on the mating-type chromosome, with HMR**a** located approximately 100-kb downstream of *MAT***a**1/*MAT*α1 and HMLα approximately 200-kb downstream of *MAT***a**2/*MAT*α2 (Figure 1.10B). However, the extent of homology between HMR**a**, HMRα and *MAT* differs. HMRa shares the X, Y**a** and Z1 sequences with *MAT***a**, while HMLα is homologous with *MAT*α for the entire region spanning W and Z2 (Figure 1.10B). By having all three loci present within the genome, *S. cerevisiae* is able to copy sequences from the silent loci and translocate these sequences to the active *MAT* locus. Thus, yeast are able to switch mating types and both *MAT***a** and *MAT*α cell types are present within any given colony (Haber, 1998). Mating-type switching ensures that compatible cell types are always within close proximity for mating to generate the more robust, diploid cell type. The formation of diploids following mating between two haploid strains of *S. cerevisiae* permits this fungus to undergo meiosis and spore formation.

1.5.2 Mating type in *Coprinus cinereus* and *Schizophyllum commune*

In the mushroom fungi *C. cinereus* and *S. commune*, the mating-type loci are designated *A* and *B*. Both fungi are tetrapolar because four different loci govern compatibility during mating interactions and therefore, segregation of the loci can result in sexual progeny with four different mating types. (Casselton and Olesnicky, 1998)

In *U. maydis*, the *a* locus of encodes pheromones and pheromone receptors, while a pair of genes encoding homeodomain proteins are present at the *b* locus. A more detailed description of the *U. maydis* mating-type loci is presented above (see section 1.3.3). Similar to *U. maydis*, the mating-type loci in the mushroom fungi contain genes encoding either pheromones and receptors or transcription factors, however the designation of *A* and *B* to these loci is opposite to *U. maydis*. Furthermore, the mushroom fungi have much more complex mating-type loci in

comparison to the tetrapolar smut fungus *U. maydis*. The genes at *A* encode homeodomain proteins and there are 160 specificities for *C. cinereus* and 288 specificities for *S. commune*. Furthermore, there are 79 versions of the pheromone- and pheromone receptor-encoding *B* locus in *C. cinereus*, while 81 exist in *S. commune*. The *A* and *B* loci are also divided into subloci called α and β, and there is functional redundancy between $A\alpha$ and $A\beta$ and between $B\alpha$ and $B\beta$ (Figure 1.11). Because of the complexity of the *MAT* loci in the mushroom fungi, a vast number of alleles have been generated and over 12, 000 mating types in *C. cinereus* and 23, 000 in *S. commune* exist (Casselton and Olesnicky, 1998; Kothe, 1999).

In *C. cinereus*, the $A\alpha$ sublocus contains one set of genes encoding homeodomain proteins (the *a* set) and the $A\beta$ sublocus contain two sets (*b* and *d*; Figure 1.11). Each gene pair is functionally independent such that compatibility between just one gene pair is sufficient for a successful mating interaction to occur. The $A\alpha$ sublocus from *S. commune* contains two divergently transcribed genes termed *Y* and *Z* and the $A\beta$ sublocus contains genes with homology to *Y* and *Z* (Kothe, 1999).

A region of approximately 17 kb represents the *B* locus of *C. cinereus*. This locus is divided into three sets of functionally independent genes, with each subfamily composed of a receptor and two pheromones (Figure 1.11). Although pheromones can only be recognized by receptors from the same family, mating requires compatibility within just one of the subfamilies. The *S. commune B* locus is similar to *B* from *C. cinereus* in that the locus is composed of two subloci, $B\alpha$ and $B\beta$, which are both functionally independent and redundant (Figure 1.11). However, in *S. commune*, recombination may take place between $B\alpha$ and $B\beta$ and each sublocus contains two or three genes encoding pheromone and one encoding a pheromone receptor (Casselton and Olesnicky, 1998; Kothe, 1999). The role of pheromone signaling in homobasidiomycete fungi differs from its function in heterobasidiomycetes such as *U. maydis*

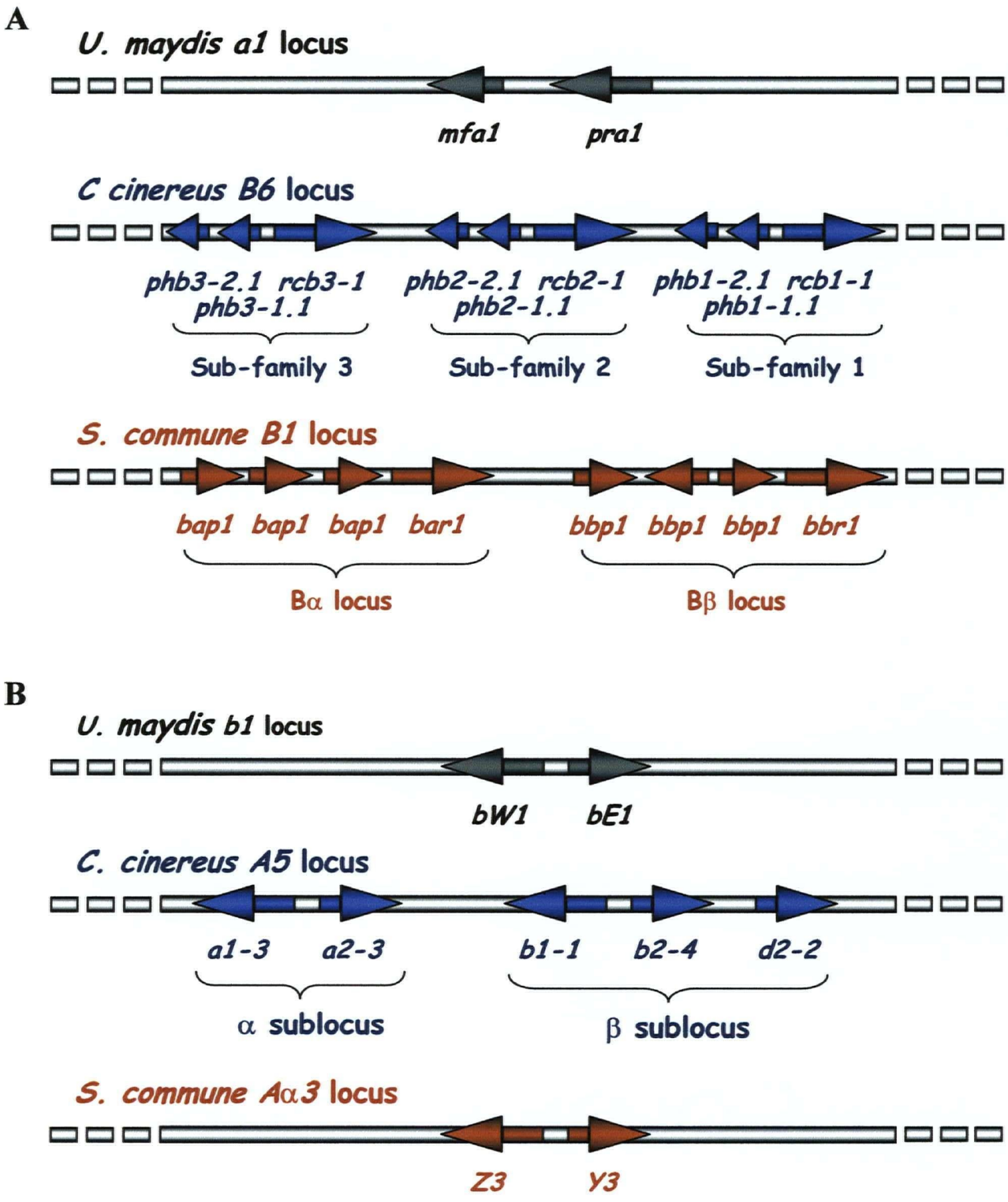


Figure 1.11. Mating-type loci from *Ustilago maydis*, *Coprinus cinereus* and *Schizophyllum commune*. The *a* locus of *U. maydis* (grey) and the *B* loci from *C. cinereus* (blue) and *S. commune* (red) contain pheromones and pheromone receptors (A). The *b* locus of *U. maydis* and the *A* loci from *C. cinereus* and *S. commune* encode homeodomain proteins (B). The mating type genes are shown as boxed arrows denoting the direction of transcription.

and *U. hordei* because cell fusion occurs regardless of mating type. Instead, pheromone signaling appears to regulate nuclear migration and growth of the dikaryon in *C. cinereus* and *S. commune*.

1.6 Research Basis and Objectives

At the time that the strategies providing the basis of this thesis were proposed, relatively little was known about the genes controlling *Ustilago* survival and proliferation within host tissue. Research in the field was focused primarily on understanding filamentous growth and the role of mating type genes in pathogenesis. A major goal was to identify targets of the major pathogenicity factor, the bE/bW heterodimer, but this proved to be quite challenging for several groups. Manuscripts describing this research are only now starting to appear in the literature, after more than 10 years of work (Brachmann *et al.*, 2001; Romeis *et al.*, 2000). In light of this slow pace, alternate approaches to identifying virulence factors in smut fungi were sought and this thesis describes two such strategies: 1) physical characterization of the *MAT* locus and 2) molecular genetic analysis of signaling pathway components. The identification of several genes necessary for pathogenesis using two indirect assays has verified the validity of these alternative approaches.

Objective 1. Characterization of the *MAT* locus: a region needed for mating and virulence in *U. hordei*.

Mating is absolutely required for the formation of the infectious cell type in smut fungi and the mating-type genes are considered pathogenicity factors. Thus, it was reasoned that the characterization of mating-type genes and the mating-type locus would reveal additional factors involved in mating and that these factors would also play a role in pathogenesis. The first

objective of this work was to characterize the unusually large mating-type locus of *U. hordei*. The results set the stage for recent investigations that provide evidence that this locus harbours multiple genes potentially involved in mating and pathogenesis (G. Jiang, personal communication).

Objective 2. Identification and characterization of factors responsible for morphogenesis in *U. maydis*

Similar to the relationship between mating and pathogenesis, morphogenesis and pathogenesis are also tightly correlated in smut fungi. This provides the foundation for the second approach to identifying virulence factors. The association between morphogenesis and pathogenesis applies not only to smut fungi, but several other fungal pathogens as well (Rooney and Klein, 2002). The term dimorphism has been used to define the ability of fungi to grow vegetatively in either a yeast or filamentous form (Shepherd, 1988). This transition has often been correlated with the switch from saprophytic to pathogenic growth stages for both plant and animal pathogenic fungi. Therefore, the second objective of this study was the identification and characterization of factors responsible for morphogenesis in *U. maydis*. It was anticipated that the identification of additional morphogenetic factors would provide new insights into the relationships between the mechanisms that regulate morphogenesis and pathogenesis. This approach identified a gene involved not only in morphogenesis and pathogenesis, but also in mating, providing further evidence of the intimate connection between these processes.

CHAPTER 2: Materials and Methods

2.1 Strains and Media

All strains employed in this study are listed in Table 2.1. Fungal strains were grown in potato dextrose broth (PDB), on potato dextrose agar plates (PDA; Difco), or on complete medium agar plates (CM; Holliday, 1974). *U. hordei* strains were grown at 22°C, while *U. maydis* strains were grown at 30°C. Fungal cells were spotted on double complete medium (DCM) agar with 1% activated charcoal for mating tests (DCM-C; Day and Anagnostakis, 1971; Holliday, 1974) or grown on DCM with 1M sorbitol after transformation. Transformants were then streaked onto CM agar containing 250 μg/ml hygromycin B (Calbiochem), 20 μg/ml phleomycin (Cayla) and/or 50 μg/ml nourseothricin (Werner BioAgents) for antibiotic selection, or inoculated into liquid CM broth with 150 μg/ml hygromycin B or 100 μg/ml nourseothricin. *Escherichia coli* strain DH5α (Gibco BRL/Invitrogen) was used for all DNA cloning experiments and strain DH10B (Invitrogen) was used for transformation by electroporation. *E. coli* strains were grown in Luria-Bertani (LB) broth or agar with 100 μg/ml ampicillin or 12.5 μg/ml chloramphenicol (Sambrook *et al.*, 1989).

2.2 DNA and RNA manipulations

Standard procedures were followed for molecular cloning as well as DNA and RNA hybridization analysis (Sambrook *et al.*, 1989). DNA restriction and modifying enzymes were obtained from Invitogen, Böehringer Mannheim, New England Biolabs and Amersham Pharmacia. DNA was introduced into *U. hordei* strains by electrotransformation (Bakkeren and Kronstad, 1993). Transformation of *U. maydis* was accomplished as described by Wang *et al.* (1988). Homologous recombination between the transforming construct and chromosomal

Table 2.1 Strains

Strain	Genotype	Source
U. hordei 549	*a2b1* (*Δb2,UhbWE1, hyg^r, phleo^r*) [b]	Bakkeren and Kronstad, 1996
U. hordei 550	*a2b1* (*Δb2,UhbWE1, hyg^r, phleo^r*) [b]	Bakkeren and Kronstad, 1996
U. hordei 551	*a1b2* (*Δb1,UhbW2, hyg^r, phleo^r*) [b]	Bakkeren and Kronstad, 1996
U. hordei 552	*a1b2* (*Δb1,UhbW2, hyg^r, phleo^r*) [b]	Bakkeren and Kronstad, 1996
U. hordei 4857-4	*a1b1* (wild type strain)	P. THOMAS; Bakkeren and Kronstad, 1996
U. hordei 4857-5	*a2b2* (wild type strain)	P. THOMAS; Bakkeren and Kronstad, 1996
U. hordei 364-62 / 364-86	*a1b1* (*Δpan1::phleo^r/SceI*) [c]	Katherine Wong
U. hordei 364-86dt21	*a1b1* (*Δpan1::phleo^r/SceI, ΔbWE1::hyg^r/SceI*) [d]	Katherine Wong
U. hordei 365-57 / 365-71	*a2b2* (*ΔbW2::hyg^r/SceI*) [e]	Katherine Wong
U. hordei 365-57dt51	*a2b2* (*ΔbW2::hyg^r/SceI, Δpan2::phleo^r/SceI*) [f]	This work
U. maydis 521	*a1b1*	R. Holliday
U. maydis 518	*a2b2*	R. Holliday
U. maydis 001prV16Hyg	*a2b2 (prV16Hyg)*	This work
U. maydis 002prV16Hyg	*a1b1 (prV16Hyg)*	This work
U. maydis 001pHyg101	*a2b2 (pHyg101)*	This work
U. maydis 002pHyg101	*a1b1 (pHyg101)*	This work
U. maydis FB1	*a1b1*	F. Banuett
U. maydis FB2	*a2b2*	F. Banuett
U. maydis FB1prV16Hyg	*a1b1 (prV16Hyg)*	This work
U. maydis FB2prV16Hyg	*a2b2 (prV16Hyg)*	This work
U. maydis FB1pHyg101	*a1b1 (pHyg101)*	This work
U. maydis FB2pHyg101	*a2b2 (pHyg101)*	This work
U. maydis FB1prV16Sat	*a1b1 (prV16Sat)*	This work
U. maydis FB2prV16Sat	*a2b2 (prV16Sat)*	This work
U. maydis FB1pSat112	*a1b1 (pSat112)*	This work
U. maydis FB2pSat112	*a2b2 (pSat112)*	This work
U.maydis d132	*a1/a2 b1/b2*	Kronstad and Leong, 1989
U. maydis d132prV16Hyg	*a1/a2 b1/b2 (prV16Hyg)*	This work
U. maydis d132pHyg101	*a1/a2 b1/b2 (pHyg101)*	This work
U. maydis 001-12	*a2b2 Δadr1::phl*	Dürrenberger *et al.*, 1998

U. maydis 001Δras1	*a2b2 Δras1-2, hyg^r*	This work
U. maydis 002Δras1	*a1b1 Δras1-2, hyg^r*	This work
U. maydis 001Δras1pSat112	*a2b2 Δras1-2, hyg^r, (pSat112)*	This work
U. maydis 001Δras1prV16Sat	*a2b2 Δras1-2, hyg^r, (prV16Sat)*	This work
U. maydis 002Δras1pSat112	*a2b2 Δras1-2, hyg^r, (pSat112)*	This work
U. maydis 002Δras1prV16Sat	*a2b2 Δras1-2, hyg^r, (prV16Sat)*	This work
U. maydis 001Δras1pX696S	*a2b2 Δras1-2, hyg^r,(pX696S)*	This work
U. maydis FB1-26	*a1b1Δfuz7, hyg^r*	F. Banuett
FB1-26pSat112	*a1b1Δfuz7, hyg^r, pSat112*	This work
FB1-26prV16Sat	*a1b1Δfuz7, hyg^r, prV16Sat*	This work
U. maydis FB1Δubc3	*a1b1Δubc3, nat^r*	Muller *et al.*, 1999
FB1Δubc3pHyg101	*a1b1Δubc3, nat^r, (pHyg101)*	This work
FB1Δubc3prV16Hyg	*a1b1Δubc3, nat^r, (prV16Hyg)*	This work
U. maydis 001Δprf1	*a2b2 Δprf1, hyg^r*	Y. Kohno, A. De Maria & N. Lee, unpublished
U. maydis 002Δprf1	*a1b1Δprf1, hyg^r*	Y. Kohno, A. De Maria & N. Lee, unpublished
U. maydis 001Δprf1pSat112	*a2b2 Δprf1, hyg^r, (pSat112)*	This work
U. maydis 001Δprf1prV16Sat	*a2b2 Δprf1, hyg^r, (prV16Sat)*	This work
U. maydis 002Δprf1prV16Sat	*a1b1 Δprf1, hyg^r, (prV16Sat)*	This work
U. maydis 002Δprf1pSat112	*a1b1 Δprf1, hyg^r, (pSat112)*	This work
U. maydis 0606	*a2b2 Δubc1, phleo^r*	Dürrenberger *et al.*, 1998
U. maydis 0606pHyg101	*a2b2 Δubc1, phleo^r, (pHyg101)*	This work
U. maydis 0606prV16Hyg	*a2b2 Δubc1, phleo^r, (prV16Hyg)*	This work
U. maydis UT0305	*a2b2 Δukc1-2, phleo^r*	Dürrenberger and Kronstad, 1999
U. maydis UT0305pHyg101	*a2b2 Δukc1-2, phleo^r, (pHyg101)*	This work
U. maydis UT0305prV16Hyg	*a2b2 Δukc1-2, phleo^r, (prV16Hyg)*	This work
U. maydis ISO1-1	*a1b1 Δukc1-2, phleo^r*	Dürrenberger and Kronstad, 1999
U. maydis ISO1-1prV16Hyg	*a1b1 Δukc1-2, phleo^r, (prV16Hyg)*	This work
U. maydis ISO1-1pHyg101	*a1b1 Δukc1-2, phleo^r, (pHyg101)*	This work
U. maydis 3020	*Δhgl1, nat^r*	Dürrenberger *et al.*, 2001
U. maydis 3020pHyg101	*Δhgl1, nat^r, (pHyg101)*	This work
U. maydis 3020prV16Hyg	*Δhgl1, nat^r, (prV16Hyg)*	This work

U. maydis 33-1 (pHyg101)	*a2b2 Δadr1, phleo^r, (pHyg101)*	This work
U. maydis 33-1 (prV16Hyg)	*a2b2 Δadr1, phleo^r, (prV16Hyg)*	This work
U. maydis 33-1 (pX6-9)	*a2b2 Δadr1, phleo^r, (pX6-9)*	This work
U. maydis 33-1 (pX696rh)	*a2b2 Δadr1, phleo^r, (pX696rh)*	This work
U. maydis P6D	*a2b2(a1b1 loci ectopically integrated)*	Giasson and Kronstad, 1995
U. maydis P6DΔras1	*a2b2 Δras1-2 (a1b1 loci ectopically integrated)*	This work
U. maydis P6D prV16Hyg	*a2b2 Δras1-2ras1^{Val16} (a1b1 loci ectopically integrated)*	This work
U. maydis 001Δras1 prV16Sat	*a2b2 Δras1-2, (prV16Sat)*	This work
U. maydis 002Δras1 prV16Sat	*a1b1 Δras1-2, (prV16Sat)*	This work

DNA was confirmed through hybridization analysis. Fungal genomic DNA was isolated by phenol extraction after disruption by glass beads (Elder *et al.*, 1983). Fungal cells were grown on DCM agar with activated charcoal for 48 hours and RNA was isolated essentially as described (Schmitt *et al.*, 1990) with the exception that the fungal cells were incubated with phenol at 65°C for 15 min. In addition, the RNA was extracted with phenol/chloroform/isoamyl alcohol for a total of four times. For DNA hybridization analysis, gels were stained with ethidium bromide, treated with 0.25 M HCl for 10 min and the DNA was transferred to nylon membranes (Hybond-N+, Amersham Pharmacia Biotech). For RNA hybridization analysis, 15 μg of total RNA was loaded into each lane and the RNA was subjected to electrophoresis through a formaldehyde gel, (Sambrook *et al.*, 1989) equilibrated in 1X PO_4 buffer (25 mM Na_2HPO_4/25 mM NaH_2PO_4; pH 7.0) and transferred to nylon membranes in the same buffer. The nylon membranes were either baked for 1 hr at 80°C or cross-linked using ultraviolet light (UV Translinker TL-2000). For all hybridizations, the DNA probes were labelled with [α-^{32}P]dCTP by random-priming (Amersham Pharmacia Biotech). The DNA fragments used as hybridization probes are listed in Table 2.2. Prehybridizations and hybridizations were in 7% SDS, 0.5 M Na_2HPO_4 at 65°C and membranes were washed in 0.1X SSC (15 mM NaCl/1.5 mM sodium citrate, pH 7.0)/0.1% SDS (sodium dodecyl sulfate). The membranes were exposed to film (Kodak XAR5) with an intensifying screen at -70°C.

2.3 *Ustilago hordei* procedures

2.3.1 Plasmid constructions and gene complex tagging

Four DNA constructs were used to tag the *a* and *b* gene complexes from *MAT-1* and *MAT-2* strains. It should be noted that the fragments used to tag the *a1*, *b1* and *b2* gene complexes and the corresponding strains tagged at these gene complexes were made by Dr. Katherine Wong. In addition, the construct used to tag the *a2* gene complex was made by Dr.

Table 2.2 DNA Fragments Used For Hybridization Analysis

Probe Name	Location	Size	Enzymes used to release fragments
ae-1	*U. hordei a1* gene complex	1.9 kb	*Bss*hI/*Hin*dIII
a1sp	*U. hordei a1* gene complex	2.2 kb	*Sac*I/*Eco*RI
aw-1	*U. hordei a1* gene complex	1.5 kb	*Bgl*II/*Bss*hII
mfa2-2	*U. hordei a2* gene complex	200 bp	*Pst*I/*Sac*I
a2-R	*U. hordei a2* gene complex	900 bp	*Xba*I/*Eco*RV
a2-L	*U. hordei a2* gene complex	700 bp	*Pst*I/*Sac*I
b1-1	*U. hordei b1* gene complex	7 kb	*Kpn*I
be	*U. hordei b1* gene complex	1.2 kb	*Sph*I/*Kpn*I
bw	*U. hordei b2* gene complex	750 bp	*Xho*I/*Hin*dIII
b2RFLP	*U. hordei b2* gene complex	1.5 kb	*Bam*HI
ras1-1 (deletion)	*U. maydis ras1* locus	1.5 kb	*Ava*I/*Hin*dIII
ras1-2 (transcript)	*U. maydis ras1* locus	0.9 kb	*Hin*dIII/*Ava*I
mfa1	*U. maydis mfa1* locus	680 bp	*Eco*RV
hgl	*U. maydis hgl1* locus	0.9 kb	*Bgl*II
cbx1	*U. maydis cbx1* locus	2.3 kb	*Sac*I
adr1	*U. maydis adr1 locus*	1.05 kb	*Pst*I/*Xho*I

Guus Bakkeren; this construct was then used during the course of the work described here to make strain 365-57dt21. The constructs are described below to document the position of the key DNA sequences including the I-*Sce*I site and selectable markers.

The four DNA fragments used to tag the *a* and *b* gene complexes in *U. hordei* were based on the plasmids pSceI-Hyg#1 and Sce-phleo#4 constructed by Dr. Katherine Wong. (Wong, 1996) To construct pSceI-Hyg#1, a 2.7-kb *Bam*HI to *Xho*I fragment containing the hygromycin B cassette was ligated with the *Bam*HI digested cloning vector pGEM3+ (Promega) and two annealed oligonucleotides containing the 18-bp I-*Sce*I recognition sequence (5'TAGGGATAACAGGGTAAT3'). The I-*Sce*I oligonucleotides SCE1 and SCE2 contain the I-*Sce*I recognition sequence placed between *Bam*HI and *Xho*I restriction sites (Table 2.3). Plasmid pSce-phleo#4 was constructed by isolating the 3.2-kb *Xho*I and *Sal*I digested vector from pHyg101 and inserting a 2.1-kb *Sal*I fragment from pUble10 (Gold *et al.*, 1994b) containing the phleomycin cassette. I-*Sce*I is an intron-homing enzyme from *S. cerevisiae* (Böehringer Mannheim; Thierry and Dujon, 1992).

The fragment used to tag the *a1* gene complex was constructed by cloning the 9.5-kb *Bss*hII fragment from paMAT-1, (Bakkeren and Kronstad, 1994) containing the *pan1* gene (encoding an enzyme required for pantothenic acid biosynthesis) and the *pra1* gene (encoding a pheromone receptor), into the vector pBSII(KS). This plasmid was then digested with *Sac*II and *Xba*I, and the ends were made blunt using T4 DNA polymerase, (the *pan1* gene was deleted). The modified pBSII(KS) plasmid was then ligated to the 2.1-kb blunt-ended *Bam*HI to *Sal*I fragment from Sce-phleo#4. *U. hordei* strain 4857-4 was then transformed using the linear 9.5-kb *Bss*hII fragment from pa1-Sce-Phleo-Δpan1-1#2 (Figure 2.1A in blue). Plasmid pUhpra2-SceI-Phleo-I was used to tag the *a2* gene complex (Figure 2.1B in blue). This construct was made by replacing the 200-bp *Pst*I to *Eco*RV fragment within the *pra2* gene (pheromone receptor) with the phleomycin resistance-I-SceI cassette. pSce-Phleo#4 was

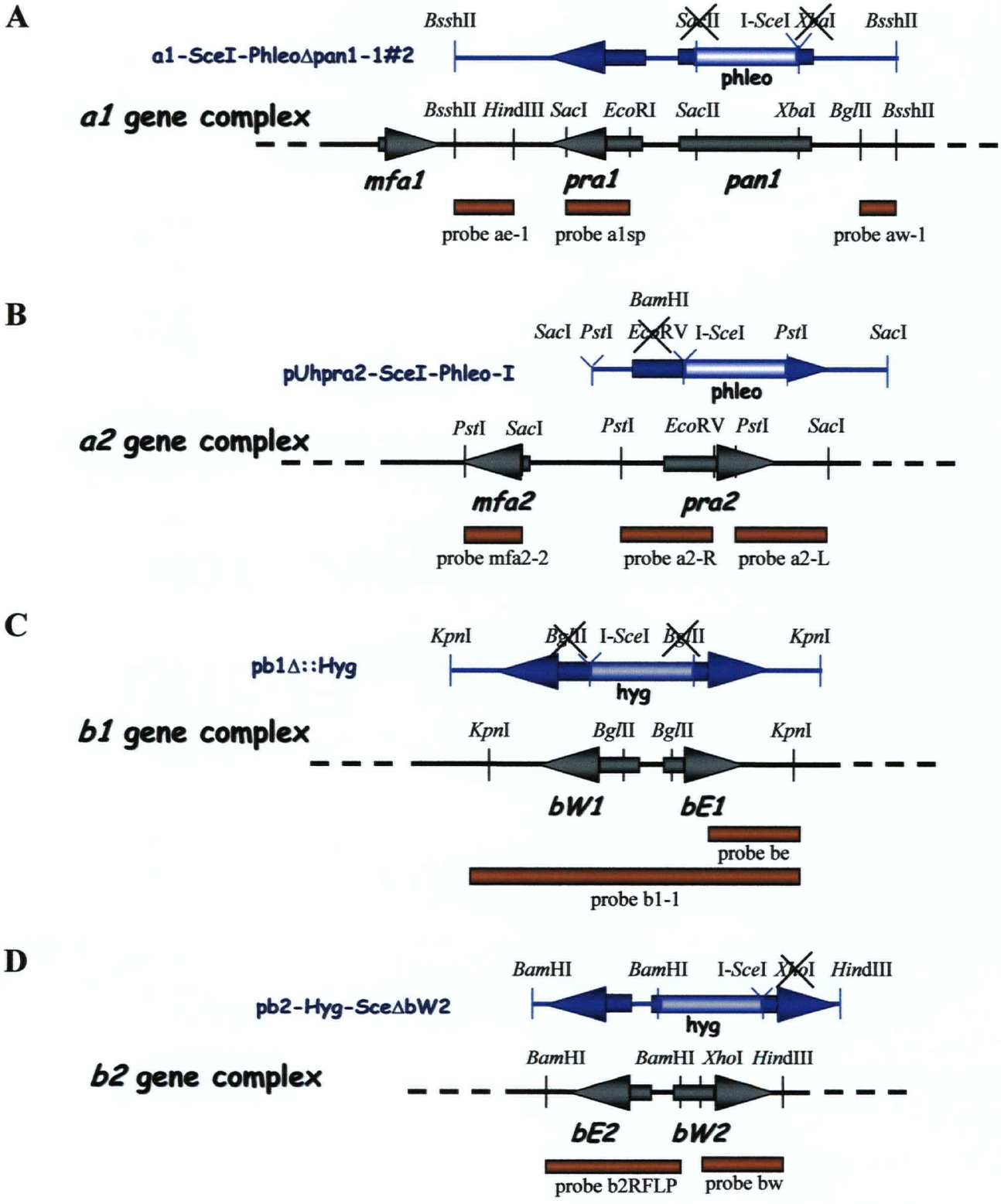


Figure 2.1. DNA constructs used for transformation and hybridization analysis in *Ustilago hordei*. The four black lines represent the *a1*, *a2*, *b1* and *b2* gene complexes. The blue lines represent inserts of plasmid constructs used to tag the respective gene complexes. The red boxes represent DNA fragments used as hybridization probes. The *mfa*, *pra*, *bE* and *bW* ORFs are shown as blue boxed arrows denoting the direction of transcription. Only the most useful restriction sites used for cloning are shown; sites that were damaged during plasmid construction are crossed out.

digested with *Eco*RI, blunt-ended and subsequently digested with *Pst*I. The 2.2-kb fragment containing the phleomycin resistance-I-*Sce*I cassette released from pSce-Pleo#4 was inserted into a pBSII(KS) vector carrying a 700-bp *Sac*I to *PstI* fragment from the 3' region of *pra2*. The phleomycin resistance-I-*Sce*I cassette and the 3' *pra2* fragment were released with *Bam*HI and ligated into a plasmid carrying the 5' region of *pra2* with a *Bam*HI linker attached at the *Eco*RV site. *U. hordei* strain 365-57 was then transformed with the 8.1-kb *Sac*I insert of pUhpra2-SceI-Phleo-I.

The construct, pb1Δ::Hyg, was used to tag the *b1* gene complex (Figure 2.1C in blue). This was made by replacing the 1.75-kb *Bgl*II fragment from pUhbWE1 (Bakkeren and Kronstad, 1993) containing the 5' ends of the *bE1* and *bW1* genes with a 2.8-kb *Bam*HI fragment of pSceI-Hyg#1 containing the hygromycin B resistance-I-*Sce*I cassettte. pb1Δ::Hyg was digested with *Kpn*I and the 11.2-kb insert was transformed into *U. hordei* strain 364-86.

The *b2* gene complex was tagged using pb2-Hyg-SceΔbW2 (Figure 2.1D in blue). This plasmid was constructed by inserting a 1.3-kb *Bam*HI fragment containing the *bE2* gene downstream of the hygromycin B resistance-I-*Sce*I cassette of pSceI-Hyg#1. The 4.2-kb fragment containing the *bE2* gene and the hygromycin resistance cassette was treated with T4 DNA polymerase to create blunt ends and ligated to a plasmid (pBSII(KS)) containing a 0.75-kb *Xho*I to *Hin*dIII fragment from the 3' region of *bW2*, thus deleting a portion of the *bW2* gene. pb2-Hyg-SceΔbW2#1 was then digested with *Kpn*I and *Not*I and the insert was used to transform *U. hordei* strain 4857-5.

2.3.2 Pulse-Field Gel Electrophoresis and hybridization analysis.

Chromosome-sized DNA from *U. hordei* was prepared and digested with I-*Sce*I essentially as described (Thierry and Dujon, 1992) except that 3.4 mg/ml of lysing enzyme (Sigma) was used to remove cell walls. Agarose plugs were immersed in 0.1 M diethanolamine

at 4°C overnight. The plugs were then dialyzed in 1 ml TE (10 mM Tris, 1mM EDTA, pH 8.0) three times for 60 min on ice. The TE was replaced with I-*Sce*I incubation buffer for 30 min and the endonuclease was diffused into the plugs for 60 min by adding 20 U of I-*Sce*I, 2 ng I-*Sce*I enhancer and fresh incubation buffer. Digestion was started by the addition of 1.6 µl of 1 M $MgCl_2$ (8 mM final) and the plugs were incubated at 37°C for 90 min on a shaker at 180 rpm. Agarose plugs were loaded into 1.2% (w/v) agarose gels in 0.5X TBE buffer (45 mM Tris-Borate, 1 mM EDTA). Gel electrophoresis was performed at 16°C using a contour clamped homogenous electric field (CHEF) electrophoresis apparatus (CHEF-DR II, Bio-Rad) under the following conditions: 45-sec pulse at 150 V for 48 hr (Figures 3.2 and 3.3D), 45-sec pulse at 200 V for 48 hr (Figure 3.3A) or 3600-sec ramped to 600-sec pulse at 60V for 120 hr and 600-sec ramped to 96-sec pulse at 60V for 50 hr (Figure 3.4).

The fragments used for hybridization probes were selected based upon their proximity to the engineered I-*Sce*I sites and are shown in bold throughout the text (Table 2). Specifically, sequences flanking each side of the I-*Sce*I site at each gene complex were used as probes (Figure 2.1 in red). The probes were purified twice by low melting point agarose gel electrophoresis and isolated from agarose by centrifugation through glass wool for 10 min at 4, 000 rpm.

2.3.3 Plant inoculation and teliospore isolation.

Barley seedlings (*Hordeum vulgare* L.) of cultivars Odessa and 66-2 (a gift from Dr. P. Thomas, Agriculture Canada) were inoculated with compatible haploid strains of *U. hordei*. Compatible fungal strains were mixed in a thick paste with sterile water. Barley seedlings were de-hulled and surface sterilized using 70% EtOH and sterile water. The seedlings were inoculated on and around the shoots after germination at 22°C for 48 hr. After another 48 hr incubation, five seedlings were planted per 4 inch pot in Sunshine mix (Home Depot). Plants

were grown in Conviron growth chambers (model EIS 3244) under the following conditions: 18 hr of daylight/6 hr of darkness, 18°C, 70% relative humidity.

The plants were scored for infection and harvested after 7 weeks. Teliospores were released from infected barley spikes by grinding with a mortar and pestle in 10 ml of sterile H_2O. The mixture was poured through 4 layers of cheesecloth and aliquotted into 1.5 ml centrifuge tubes. Surface sterilization was performed by adding sodium hyperchlorite to 0.06%, vortexing for 20-30 sec, centrifuging for 5 sec at 14, 000 x g and aspirating the supernatant. The diploid teliospores were then washed twice in sterile water, spread on PDA medium in 20 petri plates to a density of approximately 5, 000 teliospores/plate and allowed to germinate overnight. Metabasidia possessing an average of 12 haploid sporidia were collected from the PDA plates and resuspended in PDB broth. After vigorous vortexing, cells were plated onto CM to isolate individual meiotic progeny. The initial concentration of haploid cells was also confirmed through viable counts on CM agar. For the isolation of double resistant progeny (drp), cells were plated on CM agar containing both hygromycin B and phleomycin. Isolated colonies were picked and resuspended in sterile water. The suspension was vortexed for 5 min and spread onto CM agar containing hygromycin B and phleomycin to obtain isolated colonies.

2.4 *Ustilago maydis* procedures

2.4.1 Isolation and complementation of *adr1* suppressor mutants

To isolate yeast-like suppressor mutants, the filamentous *adr1* mutant strain was subjected to ultraviolet light or spread on DCM agar with activated charcoal without prior treatment for the collection of spontaneous yeast-like mutants. Selected suppressor mutants were transformed with a genomic DNA cosmid library (Barrett *et al.*, 1993) and filamentous transformants were isolated. The transformants were grown without antibiotic selection for several passages to test the stability of the filamentous phenotype. Upon confirmation that the

filamentous phenotype was correlated with the presence of a cosmid, DNA was isolated from filamentous transformants and introduced into *E. coli* DH10B electro-competent cells. Cosmid DNA isolated from *E. coli* transformants was then re-introduced into the original yeast-like suppressor mutant to confirm the presence of transforming activity on the cosmid.

2.4.2 Isolation of the *ras1* gene

The procedure described in section 2.4.1 was used to isolate the *ras1* gene. Specifically, successful complementation was obtained for suppressor strain 33-1 with the identification of a cosmid (pcos33-4) that restored filamentous growth upon transformation. pcos33-4 was digested with *Kpn*I and three subclones in the vector pBS(KS) were obtained. All three subclones were able to restore filamentous growth to 33-1 upon transformation; one clone (pKS11) was selected for further analysis because it contained the smallest insert (11 kb). Subsequent digestion of pKS11 with *Xba*I, subcloning of the fragments and retransformation of strain 33-1 resulted in the identification of a 6-kb fragment (pX6-9) with complementing activity. The Genome Priming System (GPS-1, New England Biolabs) was used to insert a transposon into pX6-9 at random intervals (as identified by restriction enzyme digestion). Sequence analysis using the universal priming sites located within the transposon allowed the sequence of a 0.65-kb region to be determined. BLAST analysis with this sequence identified *ras1* as the complementing gene.

2.4.3 Nucleotide sequence analysis of the *ras1* gene

A genomic clone from an *U.maydis* cosmid library (Barrett *et al.*, 1993) was used to sequence the wild-type *ras1* allele. The *ras1* locus was sequenced by primer walking using the primers listed in Table 2.3. The locations of these oligonucleotides within the *ras1* locus are indicated in Figure 2.2. Primers prras4 and prras5 were used to isolate the *ras1* allele from strain 33-1 (Table 2.3, Figure 2.2). A Perkin-Elmer 480 thermal cycler was used to amplify the *ras1*

TABLE 2.3. OLIGONUCLEOTIDE SEQUENCES

Sequence Name	Sequence
SCE1	5'-GATCCTAGGGATAACAGGGTAAT-3'
SCE2	5'-TCGAGATTACCCTGTTATCCCTA-3'
prras1	5'-GATGCGCAAGCGCTTGCCCC-3'
prras2	5'-GTGGACGGGTGAAGCGGCG-3'
prras3	5'-GGAGGGGCAAGCGCTTGCGC-3'
prras4	5'-CGAGAGAATGCAAGAGCC-3'
prras5	5'-GCACACACACAGCGCGG-3'
prras7	5'-AAGCTTGTGGTGCTGGGAGATGTAGGTGTAGGAAAGACG-3'
prras8	5'-CCGATGGAGACTCCGCGC-3'
prras9	5'-GCACATGCCGTCGTCGCTGCC-3'
prras10	5'-CGGGCTCGAGGAGCCAGAGCG-3'
prras11	5'CGCATGATCCGCGAACAGCGCG-3'
prras12	5'-CCAAGCAGAGAGCCATCGCC-3'
prras13	5'-GCCGCCTAAGCTTTCGCTCTGGG-3'
pradr1	5'-CCGCTTCTACGCGATCAAGG-3'
pradr2	5'-GGTCGAACACACGAATTCGG-3'
pradr3	5'-GGGAAGCGTTGTGATTTGCG-3'
pradr4	5'-GGTGGAGGTAGTCGATCGC-3'

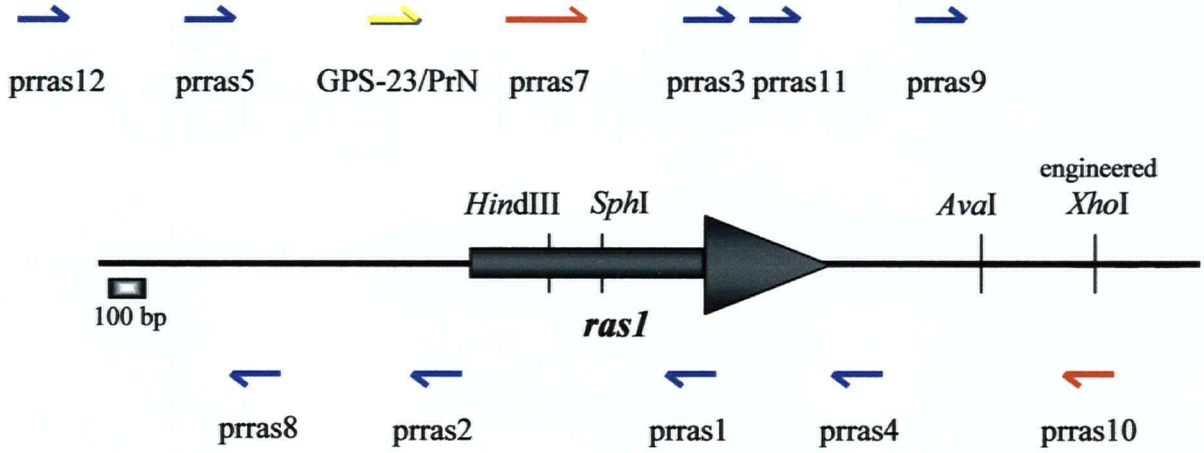


Figure 2.2 Location of the oligonucleotides used to characterize the *ras1* gene. The black line represents genomic DNA and the grey boxed arrow denotes the direction of transcription of the *ras1* gene. The primers used to sequence the *ras1* locus are shown in blue. The oligonucleotides used to introduce site specific mutations are indicated by red arrows. The yellow arrow represents the site of insertion of the transposon initially identifying the *ras1* gene.

locus using the high fidelity Vent polymerase (New England Biolabs) and the following program: 5-min. time delay at 94°C; 30-step cycles of 1-min. at 94°C, 1-min. at 65°C, and 1-min. at 72°C; 10-min at 72°C. The products of three independent PCR reactions were cloned into pBluescript KS and sequenced. Primers prras4 and prras5 were used to sequence the *ras1*^{Val116} allele from prV16Hyg (see below). Sequence alignment was performed using ClustalW (Thompson *et al.*, 1994)and presented with Boxshade 3.21.

For the identification of Ras family homologs, total genomic DNA was isolated from wild-type strain 518, digested with several enzymes (*Bam*HI, *Bgl*II, *Bss*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Kpn*I, *Mse*I, *Pst*I, *Sac*I, *Sac*II, *Sal*I, *Sph*I, and *Xba*I), transferred to a membrane and analyzed by hybridization at 37°C. The blot was washed with 2X SSC; 0.1% SDS twice for 15 minutes at 25°C for low stringency conditions. Under more stringent conditions, the blot was further washed with 0.1X SSC; 0.1% SDS for 15 minutes at 48°C.

2.4.4 Plasmid constructions

To construct deletion and activated alleles of the *ras1* gene, several plasmids were made (Figure 2.3). A 6.2-kb genomic *Xba*I fragment containing the *ras1* gene was cloned into pHyg101 and pSat112 to make pX6-9 and pX696S, respectively, for transformation into *U. maydis*. The construction of an activated *ras1* allele and *ras1* deletion alleles was based on plasmid pX696, which carries the same 6.2-kb fragment containing the wild-type *ras1* gene in pBS(KS). To make the activated *ras1*^{Val116} allele, primers prras7 and prras10 (Table 2.3) were used to amplify the 3' portion of *ras1*, introduce a glycine to valine mutation at codon 16 and engineer an artificial *Xho*I site 1-kb downstream of the *ras1* ORF (Figure 2.2). The 1.5-kb product was digested with *Hin*dIII and *Xho*I and inserted into pBluescript KS to make prV16HX. The *Hin*dIII and *Xho*I digested PCR product was also ligated to a plasmid containing the 2.9-kb

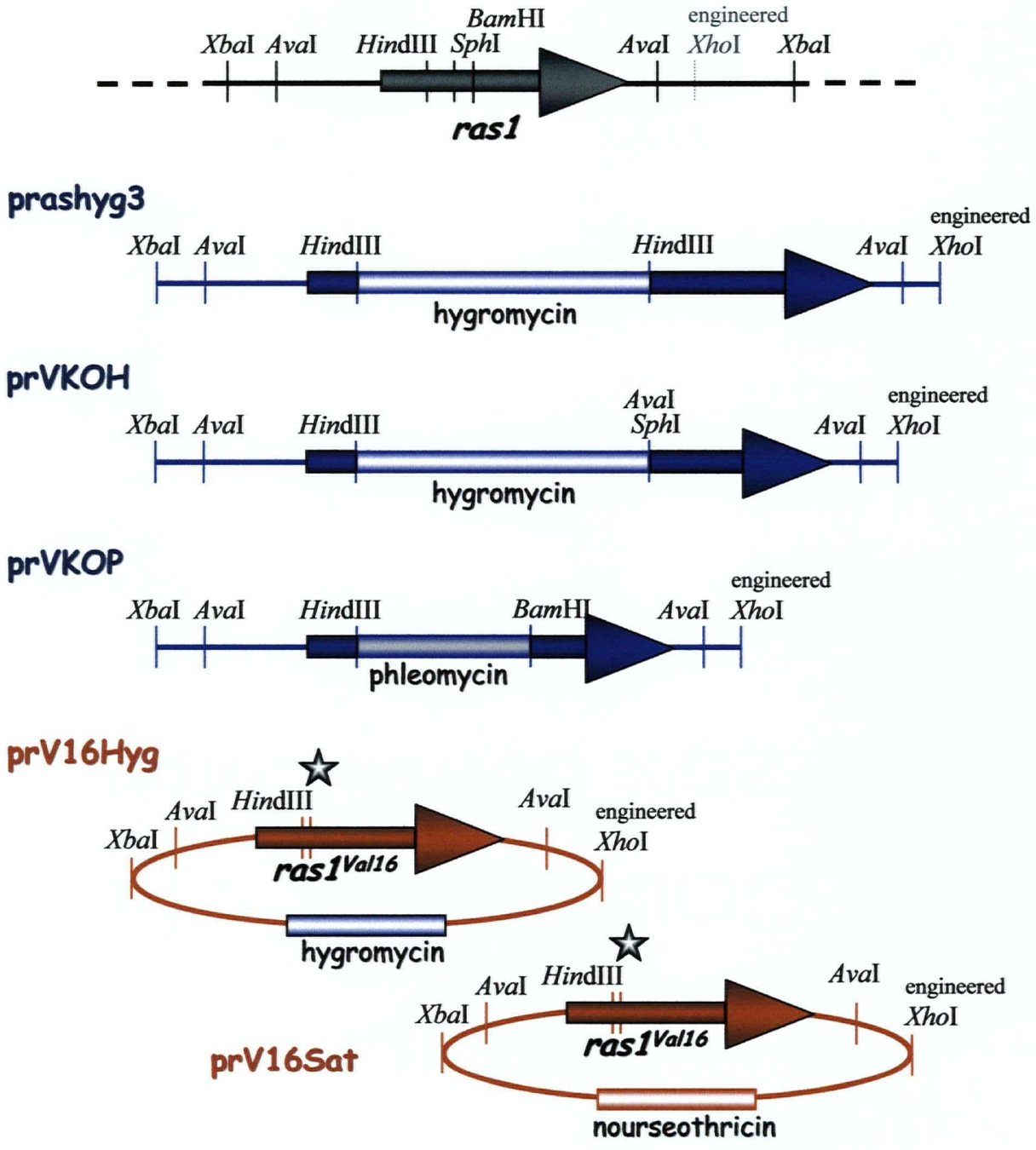


Figure 2.3. Construction of *ras1* mutant alleles. Restriction enzyme maps of the wild-type *ras1* locus (grey), the inserts of three deletion constructs (blue) and two plasmids carrying the *ras1*^{Val16} activated allele (red). The lines represent genomic DNA and the boxed arrows denote the direction of transcription of the *ras1* gene. The hygromycin resistance cassette is shown as a white box with blue tint, the phleomycin resistance cassette is a grey box with blue tint and the nourseothricin resistance cassette is represented by a white box with red tint. The grey stars in prV16Hyg and prV16Sat represent the site where the codon for glycine was replaced with valine. The *ras1* gene contains an open reading frame of 579 nucleotides.

*Xba*I-*Hin*dIII fragment of pX696 containing the 5' region of *ras1* to make prV16. For transformation into *U. maydis*, the 4.4-kb *Kpn*I-*Not*I insert of prV16 containing the $ras1^{Val16}$ activated allele was ligated into pHyg101 and pSat112 to make prV16Hyg and prV16Sat, respectively (Figure 2.3 in red). prVIH10 is a derivative of prV16 containing the hygromycin resistance cassette and a 0.3-kb genomic fragment downstream of the $ras1^{Val16}$ activated allele for integration of the activated allele into the genome at the *ras1* locus.

Three different *ras1* deletion alleles were constructed; for the *ras1-1* allele, the hygromycin resistance cassette was used to disrupt the *ras1* ORF and the *ras1-2* and *ras1-3* alleles were created by replacing a portion of the *ras1* coding region with either the hygromycin or the phleomycin resistance cassette (Figure 2.3 in blue). To make prashyg3, the *ras1* gene was disrupted by the insertion of the 2.7-kb *Hin*dIII fragment of pSceHyg#1 containing the hygromycin-resistance cassette at the unique *Hin*dIII site, correlating with codon 9 of the *ras1* gene. prashyg3 was then digested with *Kpn*I and *Not*I and inserted into pHyg101 to make plasmid pX696rh. Thus, the *ras1-1* allele represents a mutation caused by transformation with the insert of pX696rh. Plasmids prVKOH and prVKOP are similar to prV16 except that codons 9-55 or 9-75 of the *ras1* gene have been replaced by the hygromycin-resistance and phleomycin-resistance cassettes, respectively. To construct prVKOH, prV16HX was first digested with *Sph*I and *Xba*I, treated with phosphatase, and then ligated with the 2.7-kb *Xba*I-*Hin*dIII fragment of pX696 containing the 5' region of *ras1* and the 2.7-kb *Hin*dIII-*Sph*I fragment of pSceHyg#1 containing the hygromycin resistance cassette (Figure 4.6). prVKOP was constructed in a similar manner except that prV16HX was digested with *Bam*HI and *Xba*I and mixed with the 1.9-kb *Hin*dIII-*Bam*HI fragment of pScePhleo#4 containing the phleomycin resistance cassette and the 2.7-kb *Xba*I-*Hin*dIII fragment of pX696. To replace the *ras1* gene with the any of the *ras1-1*, *ras1-2* or *ras1-3* alleles, plasmids pX696rh, prVKOH or prVKOP were digested with *Kpn*I and *Not*I and transformed into *U. maydis*.

2.4.5 Mating and pathogenicity assays

Strains were tested for the production of aerial hyphae during mating reactions by spotting five µl of an overnight culture onto DCM-C agar (Holliday, 1974). The plates were wrapped in parafilm and incubated at 25°C for 48 hours. To investigate pheromone production and pheromone response, confrontation assays were performed essentially as described by Mayorga and Gold (1999), except that five ml-overnight cultures were concentrated by centrifugation and resuspended in 0.5 ml of PDB before the cells were spotted onto H_2O agar (2% agarose). Several independent assays were performed and in total, approximately fifty different interactions between each of the different strains were observed. For *U. maydis* pathogenicity assays, maize seedlings were inoculated and disease symptoms evaluated as described (Kronstad and Leong, 1989).

2.4.6 Microscopy

To document cellular morphologies, cells were grown in CM broth with the appropriate antibiotics to mid logarithmic phase and photographed with a Zeiss Axiophot microscope using differential interference contrast (DIC) optics. The morphology of *U. maydis* colonies was recorded using a Nikon Coolpix 990 digital camera mounted on a Nikon SMZ1500 microscope. On average, eight different transformants of each strain constructed were observed for phenotypic verification and photographic documentation of each strain was performed repeatedly for reliability.

CHAPTER 3: Analysis of the mating-type locus of *U. hordei*

3.1 Introduction

In smut fungi, mating is a critical prerequisite to infection. The intimate relationship between these two processes provides the basis for the work described here. Specifically, the mating-type system of *U. hordei* was characterized to provide a comparison with the mating system of *U. maydis* and to identify possible pathogenicity factors encoded at the *MAT* locus.

In basidiomycete fungi, sexual compatibility refers to the ability of two sporidia to fuse and form a dikaryon. One means by which fungal mating systems have been classified is based on the number of genetic factors that regulate the mating process (Holton *et al.*, 1968). By pairing different combinations of meiotic progeny and scoring for successful interactions, two mating-type systems have been defined: 1) bipolar systems in which compatibility is regulated by a single factor (unifactorial) with two specificities and 2) tetrapolar systems which require four different specificities at two unlinked loci (bifactorial). A third mating system with multiple specificities for a single locus has also been described for *Tilletia controversa* (Hoffman and Kendrick, 1965). Classical analysis of mating in *U. maydis* revealed a tetrapolar mating system and the two loci governing sexual compatibility were defined as *a* and *b* (Holton *et al.*, 1968). Similarly, the *MAT* locus with specificities *MAT-1* and *MAT-2* was determined to control mating in the bipolar smut fungus *U. hordei* (Yoder *et al.*, 1986).

To further investigate the difference between bipolar and tetrapolar mating systems, a molecular approach was taken to identify and characterize the genes responsible for mating in *U. maydis* and *U. hordei*. The discovery of genes encoding pheromones and pheromone receptors at the *a* locus and of genes encoding putative homeodomain proteins at the *b* locus of *U. maydis* were significant contributions to the understanding of the mechanisms of cell recognition and cell fusion during mating (Bölker *et al.*, 1992; Froeliger and Leong, 1991; Gillissen *et al.*, 1992;

Kronstad and Leong, 1989; Kronstad and Leong, 1990; Schulz *et al.*, 1990). More recent studies using DNA hybridization analysis with the genes located at the *a* and *b* mating-type loci from *U. maydis* revealed that *U. hordei* possesses similar mating-type functions located at *a* and *b* gene complexes within the *MAT* locus (Bakkeren *et al.*, 1992; Bakkeren and Kronstad, 1993). In addition, *a* and *b* have been shown to be physically linked on the largest chromosome of *U. hordei* and, together, they encode key functions within the *MAT* locus (Bakkeren and Kronstad, 1994). A sequencing survey indicated that only the *a1b1* and *a2b2* genotypes are found (Bakkeren and Kronstad, 1994). Preliminary mapping experiments indicated that these gene complexes were >150-kb apart, yet when *MAT-1* (*a1b1*) and *MAT-2* (*a2b2*) strains were crossed, recombinant progeny with genotypes *a1b2* and *a2b1* were not found. To search for these recombinant progeny, mating tests between parental strains and their progeny were performed. In *U. hordei* and *U. maydis*, only cells of opposite mating-type, that is, having different specificities at both *a* and *b*, successfully mate and form colonies with aerial hyphae (fuz^+). Conversely, haploid strains or incompatible partners of the same mating-type form yeast-like colonies. For a cross between *MAT-1* and *MAT-2* strains of *U. hordei*, all of the mating reactions from a sample of over 2000 progeny resulted in mycelial colonies (fuz^+) when tested with parental strains, suggesting that recombination is suppressed between the *a* and *b* gene complexes within *MAT* (Bakkeren and Kronstad, 1994).

To characterize the unifactorial mating-type system of *U. hordei*, strains tagged at the *a* and *b* gene complexes were constructed in the work described here and analyzed to determine the size of the *MAT* locus. Specifically, the *MAT* locus was shown to extend over a 500-kb region and the size and organization of the locus were found to differ between *MAT-1* (500 kb) and *MAT-2* (430 kb) strains. In addition, the markers used to tag these strains were used to screen a large number of progeny to demonstrate that recombination is suppressed in the region between the *a* and *b* gene complexes.

3.2 Results

3.2.1 Construction of a strain tagged at the *a2* gene complex of *U. hordei*

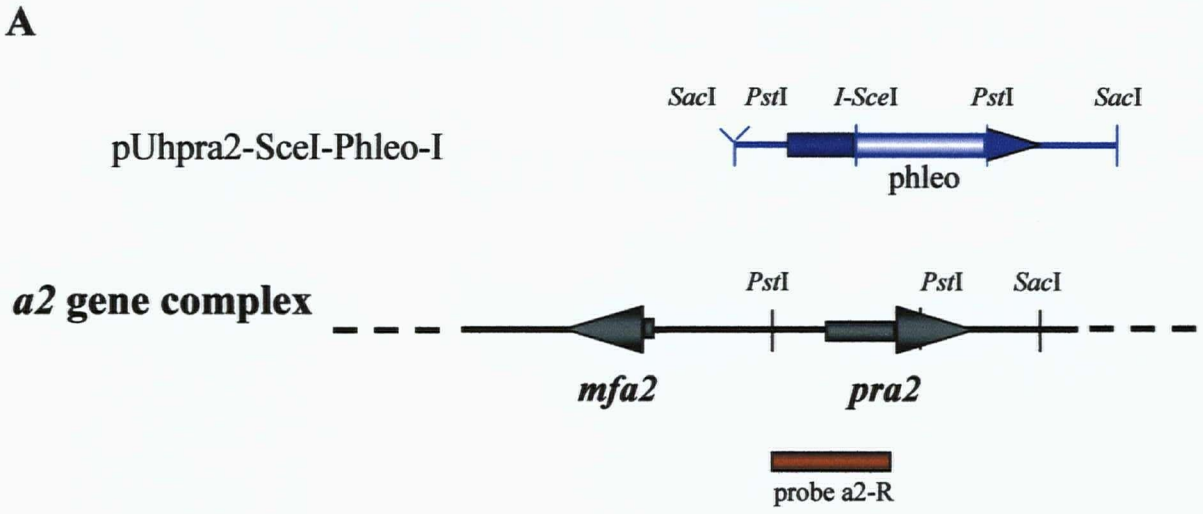
To determine the physical and genetic characteristics of the *MAT* locus of *U. hordei*, four different plasmids containing the DNA recognition sequence of the rare-cutting restriction enzyme I-*Sce*I were used to tag *MAT-1* and *MAT-2* strains at the *a1* gene complex (364-86), at the *b2* gene complex (365-57), or at both the *a* and *b* gene complexes (364-86dt21 and 365-57dt51; Table 2.1, Figure 2.1). The enzyme I-*Sce*I was chosen for these experiments because of its documented ability to specifically cleave artificially inserted recognition sequences within the genome of *S. cerevisiae* and because of its long (and therefore rare) recognition sequence (Thierry and Dujon, 1992).

In previous work, strains tagged at the *a1*, *b1* and *b2* gene complexes and a DNA construct designed to target the *a2* gene complex were made (Chapter 2.3.1). The fragment used to tag the *a2* gene complex was introduced into *U. hordei* strains 365 and 365-57 (Table 2.1). Of 220 transformants screened by hybridization analysis, only one (365-57dt51, Table 2.1) was identified as having the insert of plasmid pUhpra2-SceI-Phleo-I homologously integrated at the *a2* gene complex (Figure 3.1).

3.2.2 Physical analysis of the mating-type locus

3.2.2.1 *Determination of the distance between the a and b gene complexes*

The *MAT-1* and *MAT-2* strains tagged at both the *a* and *b* gene complexes were used to measure the physical distance between the *a* and *b* gene complexes within the *MAT* loci of *U. hordei*. Chromosome-sized DNA from the strains tagged with the recognition sequence was embedded in agarose plugs and digested with I-*Sce*I. Pulse-field gel electrophoresis and



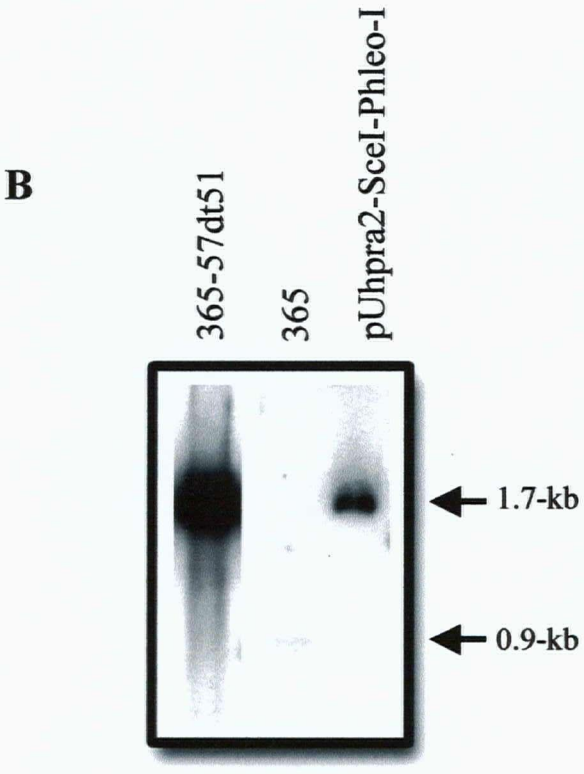


Figure 3.1 Construction and verification of a DNA fragment used to tag the *a2* gene complex. Restriction enzyme maps of the wild-type *a2* locus and the construct pUhpra2-SceI-Phleo-I (A). The black line represents genomic DNA and the grey boxed arrows denote the direction of transcription of the *mfa2* and *pra2* genes. The insert of pUhpra2-SceI-Phleo-I is drawn in blue. The location of the probe used for hybridization is shown in red. DNA hybridization analysis of the construct pUhpra2-SceI-Phleo-I, wild-type strain 365 and transformant 365-57dt51 (B). Plasmid and genomic DNA was digested with *Pst*I and hybridized with probe a2-R. Homologous integration of the phleomycin resistance-I-*Sce*I tag is indicated by the hybridization of a 1.7-kb fragment and the absence of a signal from the 0.9-kb wild-type fragment.

subsequent hybridization analysis revealed two different-sized fragments representing the regions between *a* and *b* for the *MAT-1* and *MAT-2* strains (Figure 3.2). A 500-kb fragment was released upon digestion of DNA from the *MAT-1* double-tagged strain. This band co-migrated with a 500-kb chromosome and appeared as a doublet in the gel stained with ethidium bromide (Figure 3.3A, lane 4). Hybridization with probes from the *a* and *b* gene complexes confirmed that this DNA fragment originated from the *MAT-1* locus (Figure 3.2; Figure 3.3B, lane 4; Figure 3.3C, lane 4; Figure 3.4B, lane 4). Similarly, digestion of the double-tagged *MAT-2* strain (365-57dt51) with I-*Sce*I released a fragment of ~430 kb (Figure 3.3A, lane 5). This fragment originated from the *MAT-2* locus as determined by hybridization with probes from both the *a* and *b* gene complexes (Figure 3.2; Figure 3.3B and D, lane 5; Figure 3.4E, lane 5). The 430 and 500-kb fragments were not detected by hybridization with any probe to the DNA of wild-type strains digested with I-*Sce*I, digested DNA from the single-tagged strains and undigested DNA from the double-tagged strains (Figure 3.3B-D and Figure 3.4B-C, E-F, lanes 1-3, 6-8). These control hybridization experiments support the conclusion that the 430 and 500-kb DNA fragments were indeed the regions between the *a* and *b* gene complexes.

3.2.2.2 *Determination of the chromosomal position of the MAT locus*

The DNA from wild-type, single-tagged and double-tagged strains was subjected to pulse field gel electrophoresis for an extended time (seven days versus the two days used in previous experiments) to position the *MAT* locus on the chromosome. Interestingly, both *MAT-1* and *MAT-2* are situated in the central region of an ~2.8-Mb chromosome (Figure 3.2). In *MAT-1* strains, probe **be** hybridized to ~1.6- and ~1.1-Mb fragments released upon digestion of DNA from the strain tagged only at the *a1* locus and from the strain tagged at both *a1* and *b1*, respectively (Figure 3.5D, lanes 2 and 4; see Figure 3.3A). As expected, the **bw** probe hybridized to the same 1.6-Mb fragment released from the single-tagged strain and to the ~500-

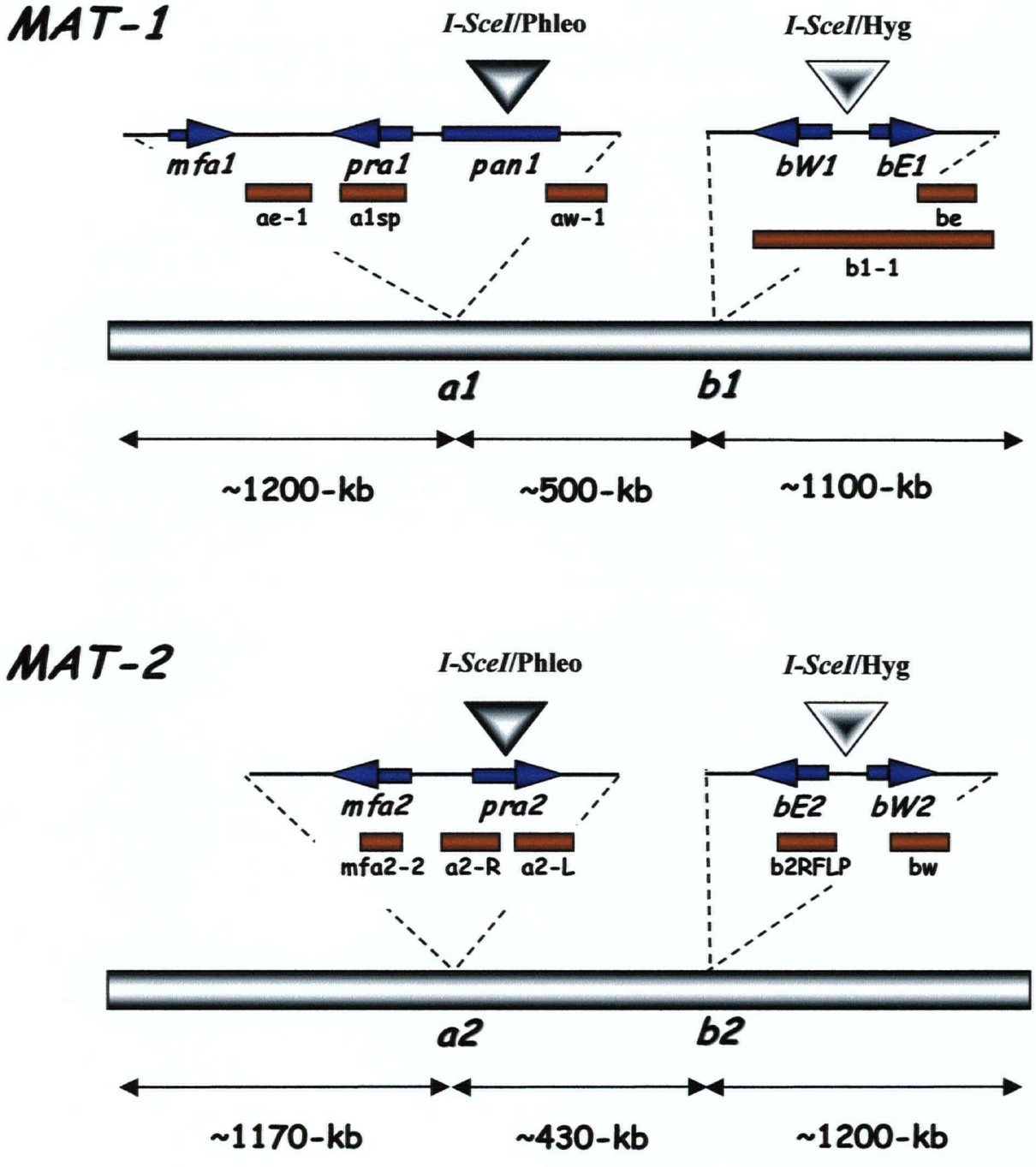


Figure 3.2 Chromosomal organization of the *MAT-1* and *MAT-2* loci. The two thick grey bars represent the *MAT* chromosomes. The thin blue lines represent inserts of plasmid constructs used to tag the respective mating-type loci. The *mfa*, *pra*, *bE* and *bW* ORFs are shown as blue boxed arrows denoting the direction of transcription. The direction of transcription of *pan1* and its location in *MAT-2* are not known. The red boxes represent DNA probes used for hybridization. See Figure 2.1 for more detail.

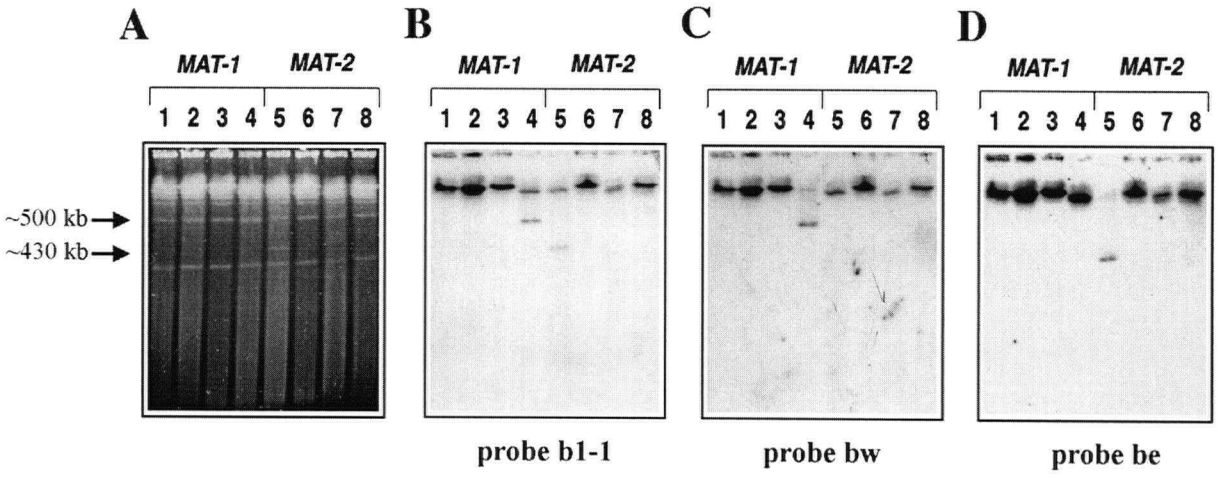


Figure 3.3 Determination of the size and organization of the *MAT* locus of *U. hordei* by hybridization with probes from the *b* gene complex. (A) Ethidium-bromide-stained CHEF gel. (B, C, D) DNA gel blots of the same gel hybridized with the probes indicated. Lanes: 1, I-*Sce*I digested 4857-4 (*a1b1*); 2, I-*Sce*I digested 364-86 (*a1b1*; single tag at *a1*); 3, undigested 364-86dt21 (*a1b1*; double tag at *a1*, *b1*); 4, I-*Sce*I digested 364-86dt21 (*a1b1*; double tag at *a1*, *b1*); 5, I-*Sce*I digested 365-57dt51 (*a2b2*; double tag at *a2*, *b2*); 6, undigested 365-57dt51 (*a2b2*; double tag at *a2*, *b2*); 7, I-*Sce*I digested 365-57 (*a2b2*; single tag at *b2*); 8, I-*Sce*I digested 4857-5 (*a2b2*). Note that probes b1-1, be and bw recognize homologous sequences in both *MAT* loci. See Figure 3.2 for probe locations.

kb fragment released from the double-tagged strain (Figure 3.5C, lanes 2 and 4; Figure 3.3C, lane 4). These data confirmed that the *a1* and *b1* gene complexes are separated by ~500 kb (Figure 3.3 and 3.4). The hybridization of an ~1.2-Mb fragment with probe **a1sp** showed that the *MAT-1* locus is centrally located, with ~1.2- and ~1.1-Mb flanking the *a1* and *b1* gene complexes, respectively (Figure 3.5B, lanes 2 and 4; Figure 3.2). Similar results were obtained for *MAT-2* strains. For example, probe **be** detected the 1.6-Mb fragment from the single-tagged strain and the 430-kb fragment from the double-tagged strain (Figure 3.5D, lanes 5 and 7; Figure 3.2). Furthermore, both probes **mfa2-2** and **bw** hybridized to ~1.2-Mb fragments released from the strain tagged at both *a2* and *b2*, revealing that the *MAT-2* locus is also located in the middle of an ~2.8-Mb chromosome (Figure 3.5B and C, lane 5).

3.2.2.3 *Determination of the organization of the a and b gene complexes within MAT-1 and MAT-2*

Hybridization with probes from either side of the I-*Sce*I site inserted at *b* revealed that the sequences at this gene complex in the *MAT-1* strain were inverted compared to the homologous sequences in the *MAT-2* strain. Both the **bw** and **be** probes contain homologous DNA sequences from the *b1* and *b2* gene complexes (Figure 2.1C-D; Bakkeren and Kronstad, 1993). The **bw** probe hybridized to the 500-kb fragment that was released upon I-*Sce*I digestion of DNA from the double-tagged *MAT-1* strain, while the **be** probe hybridized to the 430-kb fragment released upon digestion from the double-tagged *MAT-2* strain (Figure 3.3C, lane 4; Figure 3.3D, lane 5). These results indicate that the *bW* gene is closer to the *a* locus than the *bE* gene in *MAT-1* strains, and the orientation of *bE* and *bW* is reversed in *MAT-2* strains (Figure 3.2).

Hybridization probes from both sides of the I-*Sce*I site at *a1* and *a2* were used to explore the overall organization of the *a* and *b* gene complexes within *MAT-1* and *MAT-2*. The **aw-1** probe (Figure 2.1A) hybridized to the 500-kb region between *a1* and *b1*, indicating that the

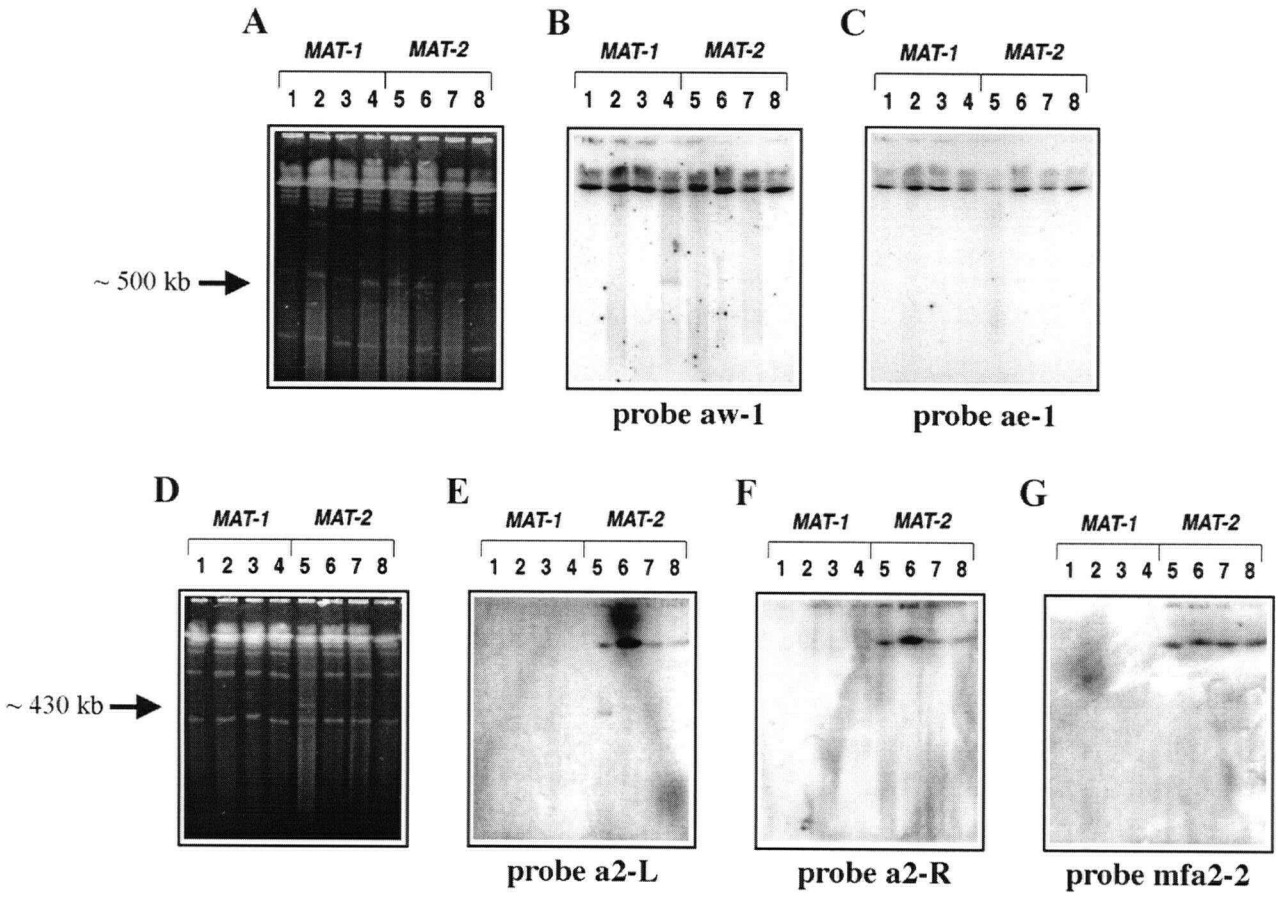


Figure 3.4. Determination of the size and organization of the *MAT* locus of *U. hordei* by hybridization with probes from the *a* gene complex. (A and D) Ethidium-bromide-stained CHEF gels. (B, C and E-G) DNA gel blots of the corresponding gels hybridized with the probes as indicated. Lanes are as described in Figure 3.3. See Figure 3.2 for probe locations.

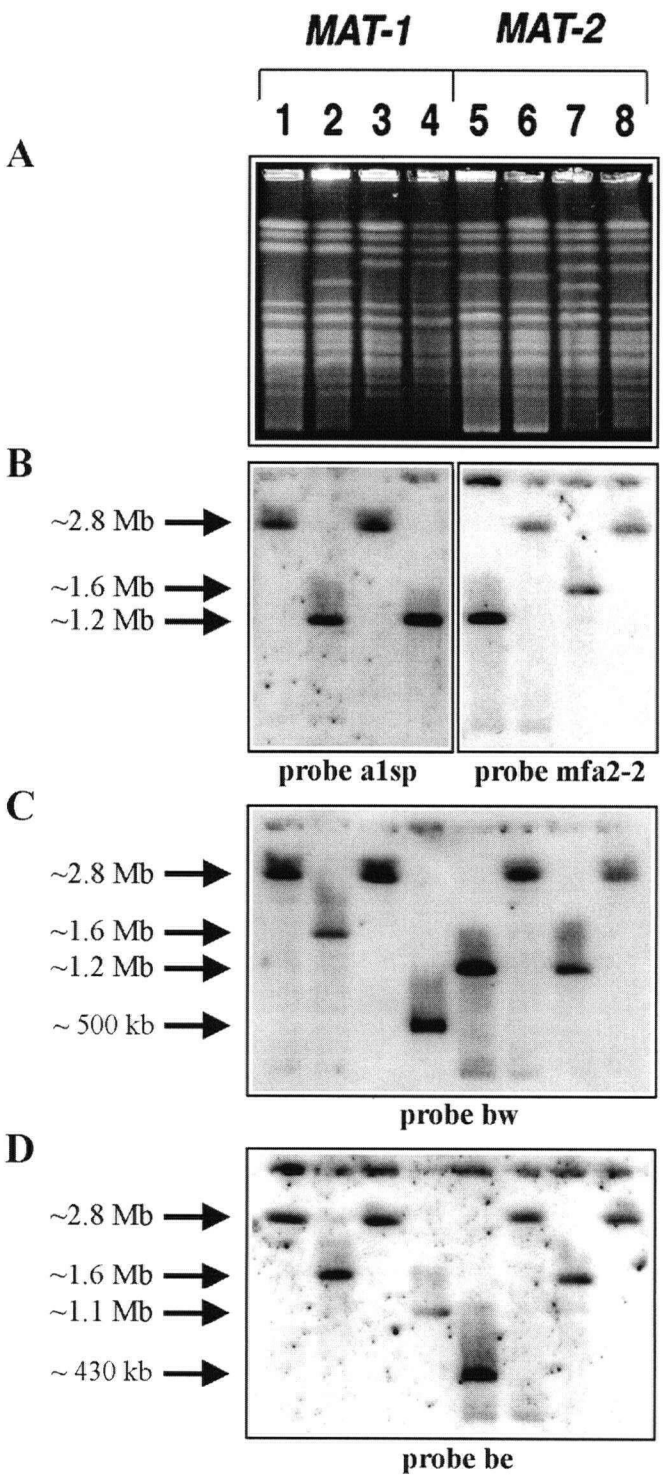


Figure 3.5. Determination of the chromosomal position of the *MAT* locus by hybridization with probes from the a and b gene complexes. (A) Ethidium-bromide-stained CHEF gel. (B, C and D) DNA gel blots of the corresponding gels hybridized with the probes as indicated. Lanes are as described in Figure 3.3. See Figure 3.2 for probe locations.

direction of transcription of *pra1* is oriented away from the *b1* gene complex (Figure 3.2; Figure 3.4B, lane 4; Anderson *et al.*, 1999). In support of this conclusion, probe **ae-1** (Figure 2.1A) hybridized to a higher molecular weight fragment shown to be ~1.2-Mb (Figure 3.2; Figure 3.4C, lane 4; comparable with probe **a1sp** in Figure 3.5B, lanes 2 and 4). For *MAT-2*, the fragment from the 3' terminus of *pra2* (probe **a2-L**, Figure 2.1B) hybridized to the 430-kb fragment released after digestion with I-*Sce*I (Figure 3.4E, lane 5). Accordingly, the probe from the 5' end of *pra2* (probe **a2-R**, Figure 2.1B) hybridized only to the higher molecular weight fragment shown to be ~1.2 Mb (Figure 3.4F, lane 5; comparable with probe **mfa2-2** in Figure 3.5B, lane 5). In addition, the hybridization with probe **mfa2-2** (Figure 2.1B) showed that *mfa2* was located upstream of *pra2* and outside of the 430-kb region spanning *pra2* and the *b* gene complex (Figure 3.4G, lane 5). The organization of the *pra* and *mfa* genes at both *a1* and *a2* has also been confirmed by PCR and sequence analysis (Anderson *et al.*, 1999). Overall, our results show that the organization of the genes at *a1* differs from that of *a2*; i.e., *pra1* and *mfa1* are convergently transcribed and *pra2* and *mfa2* are divergently transcribed.

Interestingly, the probes from the flanking sequences of the I-*Sce*I site at the *a2* gene complex (**a2-L**, **a2-R** and **mfa2-2**; Figure 2.1B) did not hybridize to DNA from *MAT-1* strains (Figure 3.4E-F, lanes 1-4; Figure 3.5B). Probe **a1sp** (Figure 2.1A), containing the *pra1* gene, was also specific for *MAT-1* strains (Figure 3.5B, Figure 3.6). These results indicate that regions of non-homology may exist between the *a* gene complexes in *MAT-1* and *MAT-2*.

3.2.3 Genetic analysis of the mating-type locus

3.2.3.1 *Determination of the frequency of recombination in the region between a and b*

The initial observation made by Bakkeren and Kronstad (1994) of an apparent low frequency of recombination in the region between *a* and *b* was further investigated by tagging the gene complexes with genes for resistance to the antibiotics phleomycin and hygromycin B.

Specifically, two strains were constructed by insertion of the phleomycin resistance gene at the *a1* locus (364-62 and 364-86) and two strains were obtained with the hygromycin B resistance gene at the *b2* locus (365-57 and 365-71; Figure 2.1, Table 2.1).

The strains tagged at the *a1* gene complex were each crossed with the two strains tagged at the *b2* gene complex by co-inoculation of barley seedlings. The goal was to estimate the frequency of recombination between the *a* and *b* gene complexes by germinating the teliospores from the crosses and selecting double-resistant recombinant progeny on a medium with both antibiotics. Presumably, only progeny having the recombinant genotype *a1b2* (*phleo*^{r}, *hyg*^{r}) would be able to form colonies, and the frequency of their occurrence would provide a measure of recombination.

To determine the frequency of recombination between the *a* and *b* gene complexes, a total of 72, 000 isolates from the four crosses (Table 3.1) were plated on a medium containing both antibiotics. Considering the number of germinated spores employed, and the number of progeny per spore (average of 12), we estimated that our sample represented between 10, 000 and 20, 000 random progeny. The selection for double-resistant progeny (drp) yielded only 34 colonies, indicating that recombination was indeed greatly suppressed within the *MAT* region, as suggested by Bakkeren and Kronstad (1994). This finding is interesting given the 400- to 500-kb distance separating *a* and *b*. The 34 drp were subsequently tested for their mating specificity in assays with two wild-type strains (4857-4, 4857-5) and four strains engineered with artificial combinations of *a* and *b* (549, 550, 551, 552, Table 2.1; Bakkeren and Kronstad, 1996). We expected that the majority of the drp would have the mating specificity *a1b2* because of the genotypes of the tagged parental strains. If this was the case, these progeny should mate exclusively with the 549 (*a2b1*) and 550 (*a1b2*) tester strains. In contrast, none of the drp mated solely with the 549 or 550 tester strains, and the majority displayed unusual mating behaviours. For example, nine of the drp displayed a constitutively mycelial phenotype on mating medium,

Table 3.1 Strains crossed for barley infection

Genotype of Strains crossed	Barley cultivar Odessa			Barley cultivar 66-2		
	# plants with teliospores	Total plants infected	% Teliospores	# plants with teliospores	Total plants infected	% Teliospores
MAT-1 hyg^r X *MAT-2 phleo*^{r}	13	46	28	30	52	58
MAT-1 hyg^r X *MAT-2 phleo*^{r}	9	37	24	17	36	47
MAT-1 hyg^r X *MAT-2 phleo*^{r}	9	37	24	31	48	64
MAT-1 hyg^r X *MAT-2 phleo*^{r}	16	36	36	28	53	53
MAT-1 X *MAT-2*	3	17	18	13	23	56

The transformants tagged at either the *a1* gene complex or the *b2* gene complex were capable of causing disease as indicated by the formation of smutted barley heads with teliospores. Teliospores were collected and germinated from each cross. Note that % teliospores refers to the percentage of barley heads containing teliospores.

suggesting the presence of both *b* specificities, and perhaps, both *a* specificities. These strains may be unreduced diploids because a constitutively filamentous phenotype is characteristic of diploid strains heterozygous at both the *a* and *b* loci in both *U. hordei* and *U. maydis* (Banuett and Herskowitz, 1989; Harrison and Sherwood, 1994; Holliday, 1961). Although numerous independent mating tests were performed, most of the progeny either failed to consistently give a positive mating reaction with any tester strain or mated routinely with more than one tester (Figure 3.6).

3.2.3.2 *RFLP analysis of the double-resistant progeny*

The possibility that the drp contained both selectable markers due to the maintenance of both parental copies of the chromosome carrying the *MAT* locus was explored by a molecular test for the presence of the *a* and *b* gene complexes from both *MAT-1* and *MAT-2*. Representative hybridization results are shown in Figure 3.6. Specifically, the **b2RFLP** hybridization probe was used to detect a restriction fragment length polymorphism (RFLP) that segregates with mating type (Figure 2.1B; Figure 3.6; Bakkeren *et al.*, 1992). Hybridization of this probe to DNA from the progeny revealed that many (13/34) contained both RFLP fragments, indicating the presence of both *b1* and *b2*. Interestingly, hybridization with *a1* and *a2* specific probes revealed that most of the drp (32/34) contained both *a1* and *a2* specific sequences (Figure 3.6). In addition, all of the drp carrying *a1* specific sequences showed an unusual hybridization pattern in that both a wild-type fragment of 5.5 kb and a unique 2.6-kb fragment hybridized to probe **a1sp** (Figure 3.6). Possibly, the extra fragment arose from a recombination event that resulted in a duplication of part of the *pra1* gene. Overall, the molecular markers demonstrated in Figure 3.6 were found in the following combinations in the 34 progeny: *a1*, *b1*, *b2* (1/34; e.g., drp#10); *mfa2*, *b1*, *b2* (1/34; e.g., drp#30); *a1*, *mfa2*, *b1*, *b2* (11/34; e.g., drp#24); *a1*, *mfa2*, *b2* (21/34; e.g., drp#13). In summary, these results indicated that

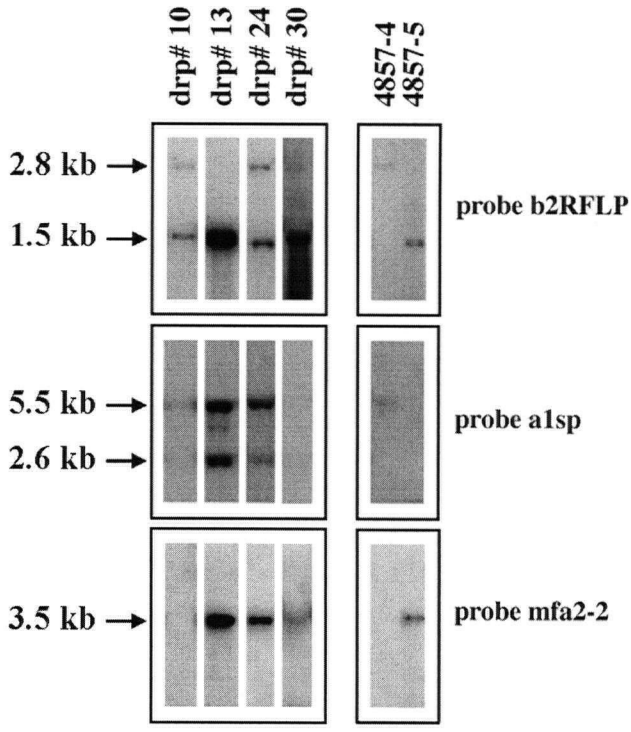


Figure 3.6 Identification of mating-type-specific sequences in the double resistant progeny (drp) by hybridization with probes from the *a* and *b* gene complexes. DNA gel blots of *Bam*HI digested genomic DNA from representative drp (see text) and both parental strains were hybridized with the probes indicated (right). The results of the hybridization analysis are summarized in the table below. See Figure 3.2 for probe locations.

drp#	MARKER				drp#	MARKER			
	a1	*mfa2*	*b1*	*b2*		*a1*	*mfa2*	*b1*	*b2*
1	√	√		√	18	√	√		√
2	√	√		√	19	√	√	√	√
3	√	√		√	20	√	√	√	√
4	√	√		√	21	√	√		√
5	√	√		√	22	√	√		√
6	√	√		√	23	√	√		√
7	√	√		√	24	√	√	√	√
8	√	√		√	25	√	√		√
9	√	√		√	26	√	√	√	√
10	√		√	√	27	√	√		√
11	√	√		√	28	√	√		√
12	√	√		√	29	√	√	√	√
13	√	√		√	30		√	√	√
14	√	√	√	√	31	√	√		√
15	√	√	√	√	32	√	√	√	√
16	√	√	√	√	33	√	√	√	√
17	√	√		√	34	√	√	√	√

the double-resistant phenotype was not the result of a simple reciprocal recombination event between *a* and *b* in the 34 drp analyzed. Rather, the drp most likely arose from other events such as rearrangements at *a* or *b*, or the retention of all or part of both *MAT* chromosome homologues to yield aneuploid, or perhaps, diploid strains. It should also be noted that true reciprocal recombinant progeny from our experiments may have been inviable and therefore not detected, although *a1b2* and *a2b1* strains have been constructed by recombinant DNA and gene replacement techniques (Bakkeren and Kronstad, 1996).

3.2.3.3 *Segregation analysis of random meiotic progeny*

To examine the possibility that the tags present in the strains crossed for the experiment shown in Table 3.1 may have interfered with meiotic crossing-over and segregation, 50 meiotic progeny were isolated from each of the four crosses. The mating type specificities of these strains were determined by plate-mating assays with two wild-type strains, 4857-4 *(a1b1)* and 4857-5 (*a2b2*). These progeny were also tested for resistance to phleomycin and hygromycin B. As shown in Table 3.2, mating type and antibiotic resistance segregated in an approximately 1:1 fashion in three of the four crosses; the fourth cross showed a reduced recovery of *MAT-2*, *phleo*^{s}, *hyg*^{r} progeny for unknown reasons. As expected, the progeny demonstrated complete linkage between phleomycin resistance and *MAT-1* mating specificity, and between hygromycin B resistance and *MAT-2*. Overall, the analysis of the 200 progeny from these crosses indicated that meiotic segregation occurred normally in the strains carrying tagged *a1* and *b2* sequences.

3.3 Discussion

3.3.1 The *MAT* locus of *U. hordei* shares similarities with eukaryotic sex chromosomes

The features of the *MAT* locus such as recombination suppression, insertion/deletions, inversion, regions of non-homology between *MAT-1* and *MAT-2* and the presence of sex-

Table 3.2 Segregation of markers in crosses to detect recombination within *MAT*

Strains crossed	Ratio of progeny with the following genotypes or phenotypes:			
	[a]*MAT-1*/ *MAT-2*	[b]phleo^r/ phleo^s	[c]hyg^s/ hyg^r	[d]phleo^r& hyg^r/ phleo^s& hyg^s
365-71 X 364-86	24/25	24/25	24/25	6/18, 400
365-71 X 364-62	23/26	23/26	23/26	7/18, 400
365-57 X 364-62	26/24	26/24	26/24	10/17, 800
365-57 X 364-86	35/15	35/15	35/15	9/17, 400
Total	108/90	108/90	108/90	34/72, 000

[a] The genotypes of the progeny were determined by performing mating tests with wild type strains 4857-4 and 4857-5.

[b] Two hundred random progeny were tested for resistance to phleomycin (phleo^r) by inoculation onto CM agar containing phleomycin.

[c] Two hundred random progeny were tested for resistance to hygromycin B (hyg^r) by inoculation onto CM agar containing hygromycin B.

[d] For each of the crosses, 20, 000 haploid progeny were inoculated onto culture medium. An average viability of ~90% was obtained for each cross, as measured by plating 100 sporidia (counted using a haemocytometer) on CM agar and counting individual colonies. (For example, for every 100 sporidia plated, 90 colonies were formed). Viability tests were performed in duplicate. A total of 72, 000 viable progeny were tested for resistance to both phleomycin (phleo^r) and hygromycin B (hyg^r) by inoculation onto CM agar containing phleomycin and hygromycin B. A second trial identified 36 progeny (out of 70, 420) that were resistant to both antibiotics.

determining genes, are reminiscent of the X/Y sex-chromosome systems (Charlesworth, 1991; Charlesworth, 1994; Jablonka and Lamb, 1990). Suppression of recombination is a common feature of mating-type loci and sex chromosomes in a variety of organisms including fungi, algae and higher eukaryotes (see Ferris and Goodenough, 1994 for a discussion). A variety of mechanisms could contribute to the suppression of recombination at mating-type loci and include nonhomology of genes at the locus, different complements of genes, chromosomal rearrangements or a special chromatin structure (Ferris and Goodenough, 1994; Kronstad and Staben, 1997). Non-homology and chromosomal aberrations are believed to have contributed to the evolution of mosaic sex chromosomes in higher eukaryotes (Charlesworth, 1994; Jablonka and Lamb, 1990). The discovery of non-homologous sequences at the *a* gene complex and inverted sequences at the *b* gene complex within the *MAT* locus of *U. hordei* provides another example of the correlation between these features of mating-type sequences and recombination suppression. It will be of interest to search the *MAT* locus for additional features that may shed light on the evolution of sex chromosomes, such as repetitive sequences and transposable elements (Charlesworth, 1994; Ferris and Goodenough, 1994). This analysis will also enhance our understanding of the mechanism by which recombination is suppressed between *MAT-1* and *MAT-2*; it may be the case, for example, that recombination suppression within the centrally located *MAT* locus may be due to the presence of the centromere. Further analysis of the chromosomes carrying *MAT* in *U. hordei* may contribute to an understanding of the evolution of dimorphic sex chromosomes and of mating-type loci in fungi.

The finding that *a1* and *a2* gene complexes contained regions of non-homology in *U. hordei* was not surprising because studies of the two *a* specificities of *U. maydis* (*a1* and *a2*) demonstrated that these sequences were idiomorphs; i.e. regions that mapped to the same chromosomal location, but contained little or no sequence homology (Bölker *et al.*, 1992; Froeliger and Leong, 1991). Furthermore, when sequences from the *U. maydis a1* idiomorph

were used to probe nitrocellulose membranes containing *U. hordei* genomic DNA, hybridization was found only to DNA from the *MAT-1* mating type (Bakkeren *et al.*, 1992). This result provided a clue that the *a* alleles of *U. hordei* were also idiomorphs. Idiomorphs were first discovered for the *A* and *a* mating-type sequences of *Neurospora crassa* (Glass *et al.*, 1990; Glass *et al.*, 1988; Staben and Yanofsky, 1990) and represent a highly conserved feature of mating sequences in this genus (Randall and Metzenberg, 1995).

3.3.2 Recombination on the *MAT* chromosome of *U. hordei*

To explore recombination suppression within *MAT*, we tagged the *a* and *b* gene complexes with antibiotic resistance markers and measured the frequency of double resistant progeny following meiosis. Although recombination was investigated only in the region between the *a* and *b* gene complexes in this study, it is conceivable that regions outside of these gene complexes may be suppressed for recombination as well. Precedent exists for suppression extending over large distances in fungi, e.g., the absence of recombination between several loci spanning almost the entire chromosome carrying the mating-type locus has been reported for *Neurospora tetrasperma* (Merino *et al.*, 1996). Very few genetic markers have been identified in *U. hordei* (i.e., for the *MAT* chromosome or for any chromosome in this fungus) and it is therefore difficult to assess the relationship between physical and genetic distance over any portion of the genome. A limited number of studies have explored recombination between the *MAT* locus and linked markers, and these indicate that crossing over occurs on the *MAT* chromosome. For example, Groth (1975) reported a recombination frequency of 10.8% between *MAT* and a gene conditioning a mycelial phenotype for haploid cells. In addition, Henry *et al.* (1988) reported a recombination frequency of 12% for the *pan-1* and *pro-2* genes in *U. hordei*; the *pan-1* gene is known to be located within or near the *a* gene complex in both *U. hordei* (Figure 3.2) and *U. maydis* (Froeliger and Leong, 1991). Additional markers at intervals along

the chromosome carrying the *MAT* locus will be needed to generate a more detailed picture of recombination frequencies across the chromosome. This information will allow us to assess the observed absence of recombination for *MAT* in the context of the whole chromosome. Further investigation may also reveal whether other factors, such as genetic regulation or the genomic organization between the *a* and *b* gene complexes, also contribute to recombination suppression within *MAT*.

3.3.3 The double resistant progeny did not arise from a simple reciprocal recombination event between *a* and *b*

The analysis of recombination within the region between the *a* and *b* gene complexes revealed a low frequency of putative recombinant progeny. It is unlikely that any of the drp that we found were true reciprocal recombinants since many of these progeny contained the *b* gene complex RFLP marker alleles from both *MAT-1* and *MAT-2* and none of these progeny could mate solely with tester strains 549 and 550 (*a2b1*). These progeny probably arose from unusual events during meiosis because many of them appear to be diploid or to contain part or all of both homologues of the *MAT* chromosome. The ability of some drp to maintain a constitutively filamentous phenotype, coupled with the appearance of both RFLP markers, lead to the conclusion that these progeny were probably unreduced diploids. The frequent appearance of diploids among meiotic progeny of the smut fungi has been documented (Harrison and Sherwood, 1994; Holliday, 1961; Puhalla, 1969).

The unusual mating behaviour exhibited by some drp (e.g., inability to mate with any tester strain or ability to mate with more than one tester) could be explained by the possibility that a fragment carrying just the *a* or the *b* sequences from one *MAT* chromosome was retained in a haploid cell of the alternate mating type, (e.g., progeny with genotypes *a1b1b2* or *a2b2b1*). Laity *et al.* (1995) found that haploid *U. maydis* strains carrying two *b* alleles were attenuated in

their ability to fuse with strains carrying a compatible *a* idiomorph. Our recovery of a low frequency of drp with unusual genetic behaviour is reminiscent of a tetrad analysis experiment performed by P. Thomas (Thomas, 1991) to assess recombination between the *MAT* locus and two auxotrophic markers in *U. hordei*. Specifically, a cross was performed between strains of genotype *pan1T481pro-1MAT-2* and *pan-1pro1T15MAT-1* and 361 ordered tetrads were dissected. All but eight tetrads had the parental combination of markers indicating tight linkage of all three traits. The remaining eight tetrads showed unusual segregation for one or more of the markers (e.g., 3:1 and 4:0 ratios) suggesting that gene conversion events may have occurred. In fact, two of these tetrads segregated 3:1 for *MAT*. In general, further characterization of the *MAT* locus will require additional physical and genetic mapping to generate a more detailed picture of recombination frequencies across the chromosome and to clarify the actual size of the *MAT* locus.

3.3.4 Does the *MAT* locus of *U. hordei* represent an eukaryotic pathogenicity island?

The experiments described in this chapter indicate that the *MAT* locus for *U. hordei* is surprisingly large compared to the mating-type sequences characterized in other fungi (Kronstad and Staben, 1997). Among fungi, the largest mating-type regions have been described in basidiomycetes. For example, the mushroom *Coprinus cinereus* possesses mating-type loci of approximately 30-kb (*A42* locus) and 17-kb (*B6* locus) (19, 20). The *A* and *B* loci of *C. cinereus* encode proteins with functional similarity to the *b* and *a*-encoded proteins of *U. hordei*, respectively. The *MAT* locus of the human pathogen *Cryptococcus neoformans* is approximately 50-kb in size and has been implicated in virulence (Karos *et al.*, 2000; Kwon-Chung *et al.*, 1992; Moore and Edman, 1993; Wickes *et al.*, 1997).

In the context of pathogenicity, the large size and multigenic nature of the *MAT* locus of *U. hordei* is reminiscent of complex pathogenicity regions that segregate as one locus in other

phytopathogenic fungi. For example, several members of the genus *Cochliobolus* are capable of synthesizing host-specific toxins, which are known to be agents of disease compatibility. The *Tox1* locus of *C. heterostrophus* and the *Tox2* locus of *C. carbonum* are responsible for the production of host-specific toxins, which promote lesion formation on leaves of susceptible host plants. Interestingly, the genetic functions for toxin synthesis segregate as a single unit although the *Tox1* locus may comprise >100-kb of DNA and *Tox2* spans a region of >500-kb (Ahn and Walton, 1996; Lu *et al.*, 1994). Races of *C. carbonum* and *C. heterostrophus* that fail to make the toxins also do not contain sequences homologous to the *Tox1* or *Tox2* loci. In *Nectria haematococca*, the genes required for pathogenicity to pea (*PEP*) have been localized to a dispensable chromosome that is proposed to be suppressed for recombination (VanEtten *et al.*, 1994). Furthermore, genes involved in the biosynthesis of elicitins are clustered in *Phytophthora cryptogea*, as are mycotoxin biosynthetic genes in *Fusarium* species, *Aspergillus nidulans* and *Gibberella fujikuroi* (Brown 1996; Desjardins 1993; Desjardins 1996; Panabieres 1995). Certainly, the clustering of genes in pathogenicity islands has been well documented in bacteria (see Lee, 1996 for a review). With the possible exception of the gene conditioning mycelial growth described by Groth (1975) other genes that may play roles in mating and pathogenicity have not yet been discovered in the *MAT* locus of *U. hordei*. The relationship between the gene described by Groth (1975) and pathogenicity remains unclear, although filamentous growth is correlated with pathogenicity in the smut fungi (Holliday, 1974).

It is tempting to speculate that other genes involved in mating and pathogenesis might be clustered along with the *a* and *b* genes at the *MAT* locus of *U. hordei*. That is, recombination suppression may serve to maintain a set of genes that function in sexual development of the fungus in the host. There is evidence for clustering of genes that function in sexual development in other basidiomycetes. For example, the *MAT*α locus of *Cryptococcus neoformans* is approximately 50-kb in size and contains several genes including pheromones, a pheromone

receptor and several mating-type α-spcific homologs of the pheromone response MAP kinase signal transduction cascade genes (Karos *et al.*, 2000). Specifically, homologs of the *S. cerevisiae STE20*, *STE11* and *STE12* genes known to be involved in regulating pheromone response were identified within the *C. neoformans MAT*α locus. In addition, the *B* locus of *Schizophyllum commune*, which encodes pheromones and pheromone receptors, is linked to a cluster of nine genes involved in nuclear migration (Raper, 1983; Wendland *et al.*, 1995). Several other loci that affect the frequency of recombination in this region are also linked to the *B* locus. The *B* locus of *S. commune* can be considered analogous to the *a* gene complex of the smut fungi because both encode pheromone signaling components. Furthermore, it is interesting to note that a number of genes that function in mating are tightly linked to the mating-type (*mt*) locus in the green alga *Chlamydomonas reinhardtii* (Ferris and Goodenough, 1994). Intrachromosomal translocations, inversions, duplications and large deletions are associated with the locus and these features are believed to account for suppression of recombination within an 830-kb stretch of DNA (Ferris and Goodenough, 1994). Although the dimorphic mating-type locus of *C. reinhardtii* has been characterized in more detail than the *MAT* locus of *U. hordei*, it is clear that these loci share common features. Further characterization of the sequences between *a* and *b* will elucidate the organization of this region, the relationship between *MAT-1* and *MAT-2* and, perhaps, reveal additional genes involved in mating and pathogenesis. As a start toward achieving these goals, probes from the *a* and *b* genes have been employed to isolate BAC clones from a *U. hordei* library constructed with DNA from a *MAT-1* strain (Appendix I). These cloned fragments will allow a more detailed view of the organization of the locus and a comparison of the mating-type loci in bipolar and tetrapolar species to gain insight into the evolutionary relationship between these systems.

CHAPTER 4: Isolation and characterization of the *ras1* gene of *U. maydis*

4.1 Introduction

In addition to mating, morphogenesis is also closely correlated with pathogenesis in *Ustilago*. The morphological transition from budding haploid cells to filamentous dikaryotic cells correlates with the switch from saprophytic growth to pathogenic development. Furthermore, competence for this morphological switch is an important factor in the virulence of several other fungal pathogens such as *Candida albicans* and *Histoplasma capsulatum* (Lo *et al.*, 1997; Maresca and Kobayashi, 2000).

One of the major factors influencing dimorphism in *Ustilago* is cAMP signaling. Mutations resulting in faulty signaling or low PKA activity lead to mutants with a constitutively filamentous phenotype (Gold *et al.*, 1994a). For example, adenylyl cyclase (encoded by the *uac1* gene) and protein kinase A (encoded by the *adr1* gene) mutants are filamentous, while PKA regulatory subunit mutants (*ubc1*) display a multiple-budding phenotype (Barrett *et al.*, 1993; Dürrenberger *et al.*, 1998; Gold *et al.*, 1994a). High PKA activity is correlated with a budding phenotype. In addition to their defects in morphogenesis, mutants deficient in the components of the cAMP/PKA pathway are avirulent, providing further evidence of a link between morphogenesis and virulence.

In an attempt to identify an effector of PKA signaling, suppressor mutants were identified that restored budding growth to the otherwise filamentous *adr1* mutant. Complementation of one of these mutants led to the identification of the *hgl1* gene (Dürrenberger *et al.*, 2001). To identify additional factors involved in morphogenesis the same genetic suppression screen that identified the *hgl1* gene was employed. Additional *adr1* suppressor mutants were isolated and the introduction of the *ras1* gene on an autonomously replicating plasmid was found to complement one of these mutants. In this chapter, evidence is presented that implicates *ras1* in

mating, morphogenesis and pathogenesis. Specifically, *ras1* was required for a basal level of pheromone production, as well as post-fusion events leading to the formation of aerial hyphae. Mutants deficient in *ras1* were altered in cell morphology and failed to induce disease symptoms upon injection into maize seedlings.

4.2 Results

4.2.1 A genetic screen for suppressors of the filamentous growth of a PKA mutant

A constitutively filamentous mutant lacking the *adr1* gene (Dürrenberger *et al.*, 1998) was used as a starting strain in a genetic suppression screen to identify targets of the cAMP pathway involved in morphogenesis. Eighty-seven suppressor mutants that demonstrated a yeast-like colony morphology were isolated. These colony morphologies ranged in phenotype from reduced filamentous growth (e.g., short filaments) to completely yeast-like morphology (Figure 4.1; Table 4.1). The suppressor mutants were classified into eight different categories based on the phenotype of colonies grown on DCM-C agar: 1) yeast-like; 2) yeast-like with sparse filaments; 3) yeast-like with intermediate filaments; 4) yeast-like with spikes; 5) colonies with short filaments; 6) filamentous colonies with yeast-like tendencies; 7) slow growing filamentous colonies; 8) filamentous colonies (slightly less filamentous than the *adr1* strain; Table 4.1). Each suppressor mutant was grown in different media (liquid PDB, on DCM agar with activated charcoal and on PDA agar) and the cellular and colony morphologies of each mutant grown in each condition were photographically documented. Interestingly, nine mutants, originally isolated due to their yeast-like phenotype, reverted back to the filamentous phenotype after several rounds of sub-culturing and growth in the different media. The phenotypic instability observed in some suppressor mutants may have resulted from second site mutations that suppressed the yeast-like morphology of the mutants. The diversity of phenotypes indicates that there may be multiple factors downstream of PKA that control filamentous growth.

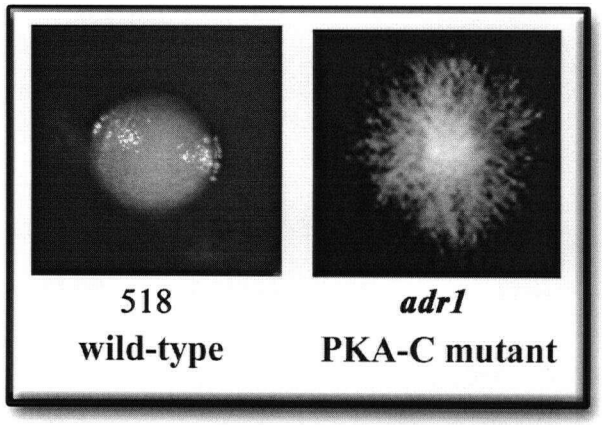
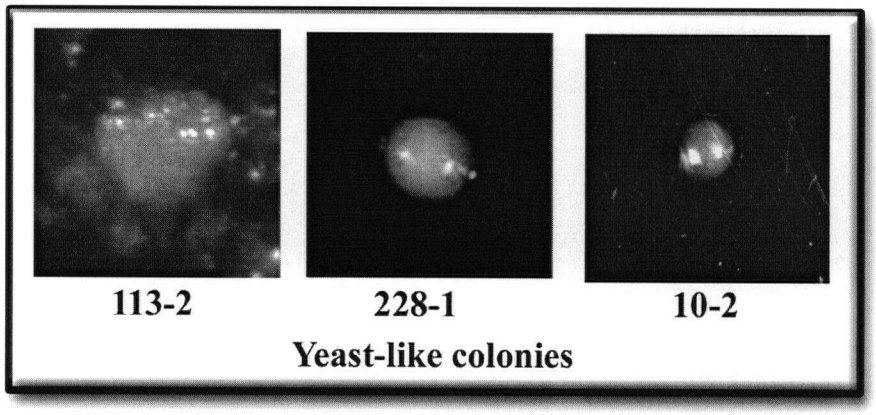
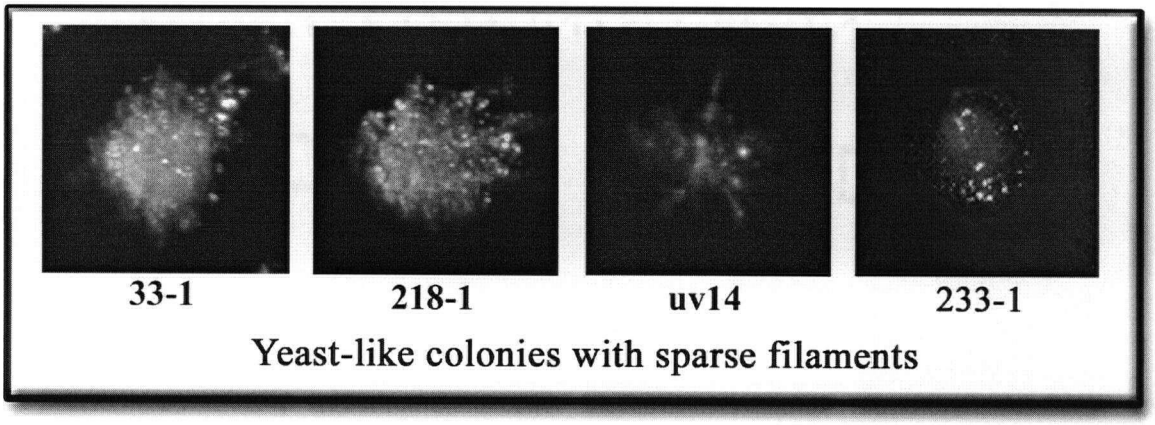


Figure 4.1 Representative colony morphologies of *adr1* suppressor mutants. Wild-type strain 518 and the *adr1* mutant are shown for comparison. The colony morphologies of suppressor mutants used in complementation studies are also shown (113-2, 228-1, 10-2, 33-1, 218-1, uv14 and 233-1). The actual sizes of the colonies are 2-4 mm.





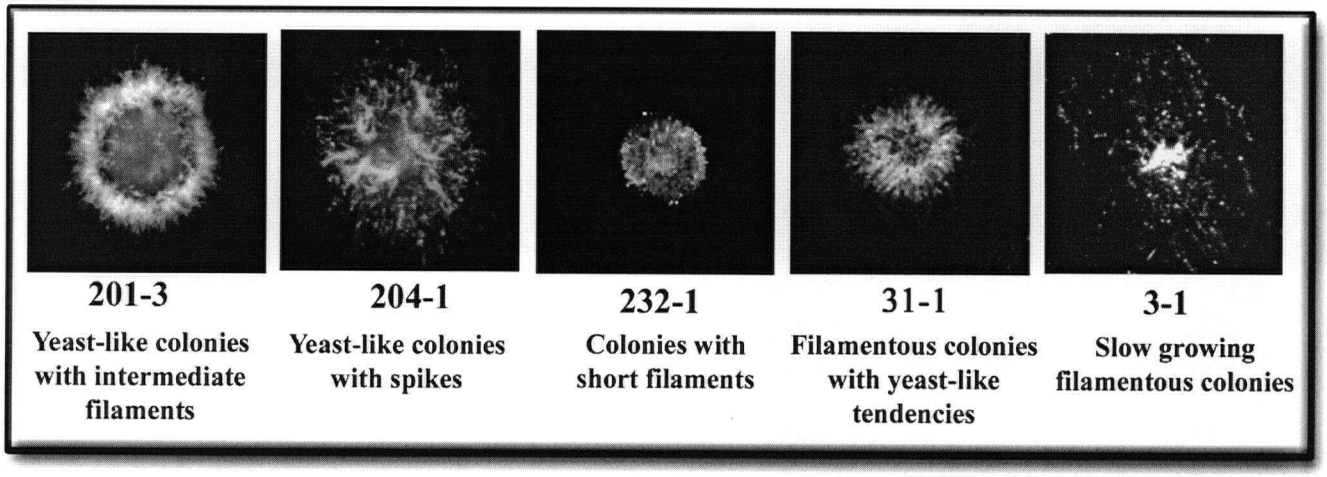


Table 4.1 Classification of *adr1* suppressor mutants

	Category	Number of mutants isolated	Suppressor mutants isolated
1	Yeast-like colonies	9	uv1, uv3, uv17, **10-2**, 13-1, **113-2**, 217-1, **228-1**, 235-1
2	Yeast-like colonies with sparse filaments	25	uv2, **uv14**, 1-1, 1-4, 12-1, **33-1**, 33-2, 33-3, 34-1, 40-1, 40-3, 41-1, 42-1, 42-3, 44-1, 44-3, 113-1, 116-1, 206-1, 206-2, 207-1, 216-1, **218-1**, **233-1**, 234-1
3	Yeast-like with intermediate filaments	13	3-2, 10-1, 12-2, 13-2, 13-3, 31-2, 44-2, 110-1, 125-1, 201-1, **201-3**, 215-1, 236-1
4	Yeast-like with spikes	7	201-2, **204-1**, 211-1, 219-1, 225-1, 230-1, 231-1
5	colonies with short filaments	3	35-1, 42-2, **232-1**
6	Filamentous colonies with yeast-like tendencies	6	**31-1**, 202-1, 203-2, 208-1, 209-1, 210-1
7	Slow growing filamentous colonies	4	**3-1**, 37-1, 39-2, 40-2
8	Filamentous colonies (<fuzzy than *adr1*)	11	uv47, 2-2, 12-3, 32-1, 32-2, 35-2, 38-1, 41-2, 45-1, 203-1, 205-1

The suppressor mutants displayed in Figure 4.1 are in bold print. The nine mutants that reverted back to a phenotype comparable to the filamentous *adr1* mutant are not listed here.

4.2.2 Complementation of selected suppressor mutants

Six suppressor mutants were chosen for further analysis with the goal of cloning the gene that was defective in each mutant. These mutants were prescreened by transformation with known genes encoding cAMP and MAP kinase pathway components involved in morphogenesis including *hgl1, ubc2, ubc3* and *ubc4* (Figure 1.6; Table 4.2). Of these six mutants, the phenotypes of three (113-2, 218-1 and 233-1) were influenced by transformation with the *hgl1* gene. For example, transformation of strain 113-2 with a plasmid carrying the *hgl1* gene resulted in filamentous transformants (indicative of complementation or copy number suppression). Introduction of the *ubc3* gene into strain 113-2 caused filamentous growth in 20-30% of the transformants suggesting that this strain may be mutated at another locus that is important for both the cAMP and MAPK pathways. To explore this possibility, strain 113-2 was transformed with a genomic library in a cosmid vector and a cosmid (pcos113-500) was isolated because of its ability to restore the filamentous phenotype to 113-2. However, DNA hybridization analysis revealed that cosmid pcos113-500 contained the *hgl1* gene (Figure 4.2). Two other suppressor mutants (218-1 and 233-1, Table 4.1) were partially complemented by the addition of a plasmid carrying the *hgl1* gene, but a cosmid that completely restored filamentous growth to these strains could not be identified. Complementation was attempted for all six selected suppressor mutants, including the three mutants (33-1, 228-1 and uv14) that were unaffected morphologically after transformation with the plasmids containing the *hgl1*, *ubc2*, *ubc3* or *ubc4* genes (Table 4.3). Successful complementation was obtained only for strain 33-1, with the identification of a cosmid (pcos33-4) that restored filamentous growth upon transformation (Figure 4.3). Subsequent subcloning of pcos33-4, retransformation of subclones into strain 33-1 and sequence analysis of the complementing region identified *ras1* as the complementing gene (see Chapter 2.4.2).

Table 4.2. Complementation of suppressor mutants with known genes

Strain	DNA Transformed				
	vector	***hgl1***	***ubc2***	***ubc3***	***ubc4***
518/521	yeast	yeast	yeast	yeast	yeast
10-2 (*hgl1*$^-$)	yeast	fil	yeast	yeast	yeast
33-1	yeast	yeast	yeast	yeast	yeast
113-2	yeast	fil (100%)	yeast	fil (20-30%)	yeast
228-1	yeast	yeast	yeast	yeast	yeast
218-1	yeast	fil	yeast	yeast	yeast
233-1	yeast	fil	yeast	yeast	yeast
uv14	yeast	yeast	yeast	yeast	yeast

Colonies displaying a yeast-like phenotype are indicated with (yeast) and transformants with filamentous phenotypes are indicated with (fil). For mutant 113-2, 100% of the transformants were complemented with *hgl1* and 20-30% of the colonies transformed with the *ubc3* gene were filamentous. The cosmid vector pJW42 was used as the vector control.

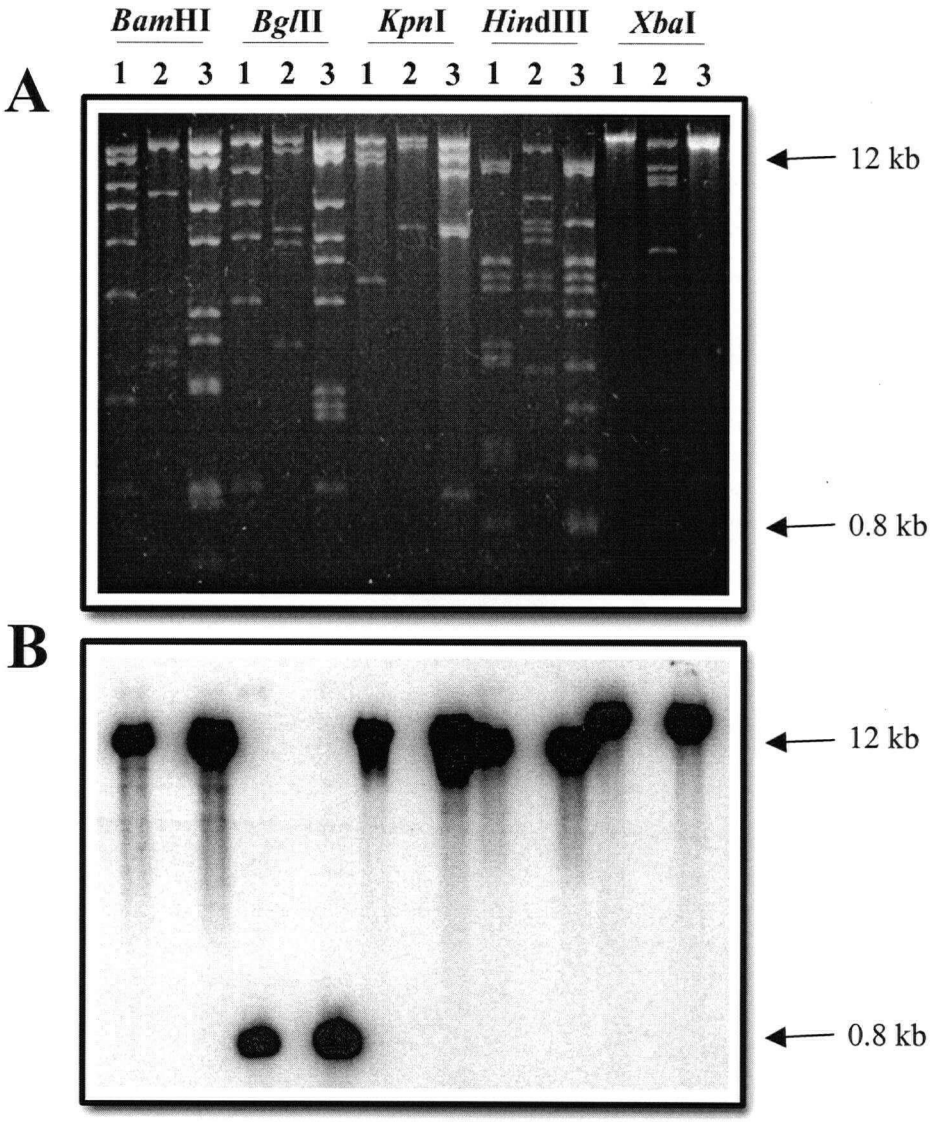


Figure 4.2 Identification of the *hgl1* gene within cosmid pcos113-500. The *hgl1* gene was originally isolated from a cosmid (pcos102) that restored filamentous growth to *adr1* suppressor mutant 10-2 (Durrenberger 2001). pcos113-500 was identified by it's ability to complement the defect in mutant 113-2. pcos102 (lanes 1), pcos33-4 (carrying the *ras1* gene; lanes 2) and pcos113-500 (lanes 3) were digested with the restriction enzymes indicated and subjected to gel electrophoresis. Ethidium bromide stained gel (A). DNA gel blot of the same gel hybridized with a fragment from the *hgl1* gene (probe hgl; Table 2.2; B)

Table 4.3 Transformation of selected suppressor mutants with cosmid and plasmid libraries

Suppressor mutant	Number of transformants screened upon transformation with:		Cosmid recovered from filamentous transformant
	cosmid library	plasmid library	
113-2	3, 000	0	pcos113-500
33-1	5, 000	2, 000	pcos33-4
228-1	9, 000	20, 000	none recovered
218-1	5, 000	10, 000	none recovered
233-1	795	500	none recovered
uv14	500	2, 000	none recovered

The cosmid library was constructed by Barrett et al (1993) and the plasmid library was made by F. Dürrenberger (unpublished).

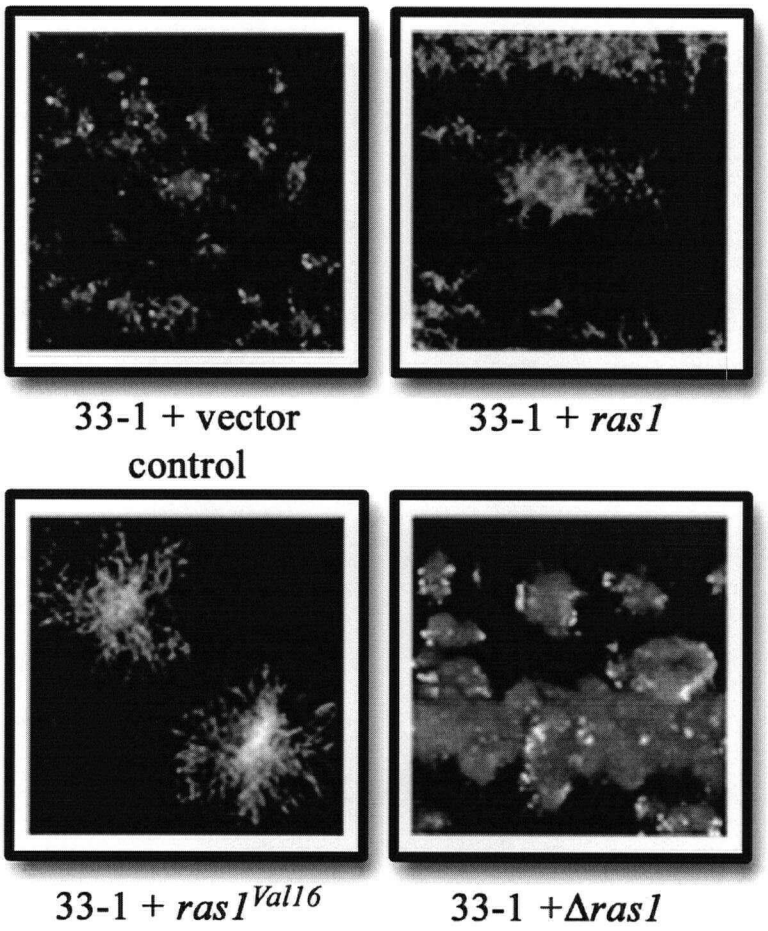


Figure 4.3. Complementation of *adr1* suppressor mutant 33-1. Colony morphologies of 33-1 transformed with a vector control (pHyg101, top left), a plasmid carrying the wild-type *ras1* allele (pX6-9, top right), a plasmid carrying the activated $ras1^{Val16}$ allele (prV16Hyg, bottom left) and a plasmid carrying a disrupted *ras1* allele (pX696rh, bottom right).

4.2.3 Characterization of the *ras1* gene of *U. maydis*

The *ras1* gene of *U. maydis* contained an open reading frame of 579 nucleotides encoding a predicted polypeptide of 192 amino acids. Ras1 had high sequence identity to other fungal Ras proteins including *Neurospora crassa* NC-ras2 (60%), *Cryptococcus neoformans RAS2* (49%), *Aspergillus fumigatus RAS* (59%), *Candida albicans RAS1* (55%), *Schizosaccharomyces pombe ras1* (53%), *Saccharomyces cerevisiae RAS2* (51%) and *Saccharomyces cerevisiae RAS1* (51%; Figure 4.4). The predicted polypeptide from the *U. maydis* gene did not contain the long carboxy terminal tail found in the Ras1 and Ras2 proteins of *S. cerevisiae*; this region is thought to mediate association with adenylyl cyclase.

To examine whether *ras1* is part of a Ras gene family, a fragment containing the *ras1* gene was used as a hybridization probe to DNA blots of genomic DNA under low stringency conditions. Although there was a high background of hybridization, several bands were detected including the major band for *ras1*, suggesting the presence of other sequences with significant homology to *ras1* (Figure 4.5; lanes 1). More stringent conditions identified a single band in each lane that represented the *ras1* gene (Figure 4.5; lanes 2). These results suggest that more than one Ras homolog may exist in the *U. maydis* genome.

4.2.4 Identification of the *ras1* gene as a copy number suppressor

The introduction of pcos33-4 and cosmid subclones containing the *ras1* gene into mutant 33-1 gave rise to transformants with variable phenotypes. Although we initially identified pcos33-4 based on its ability to restore the filamentous phenotype to colonies of 33-1, we consistently found that some of the transformants remained yeast-like (despite their resistance to hygromycin B). These results prompted an examination of the mutation in the *ras1* allele in

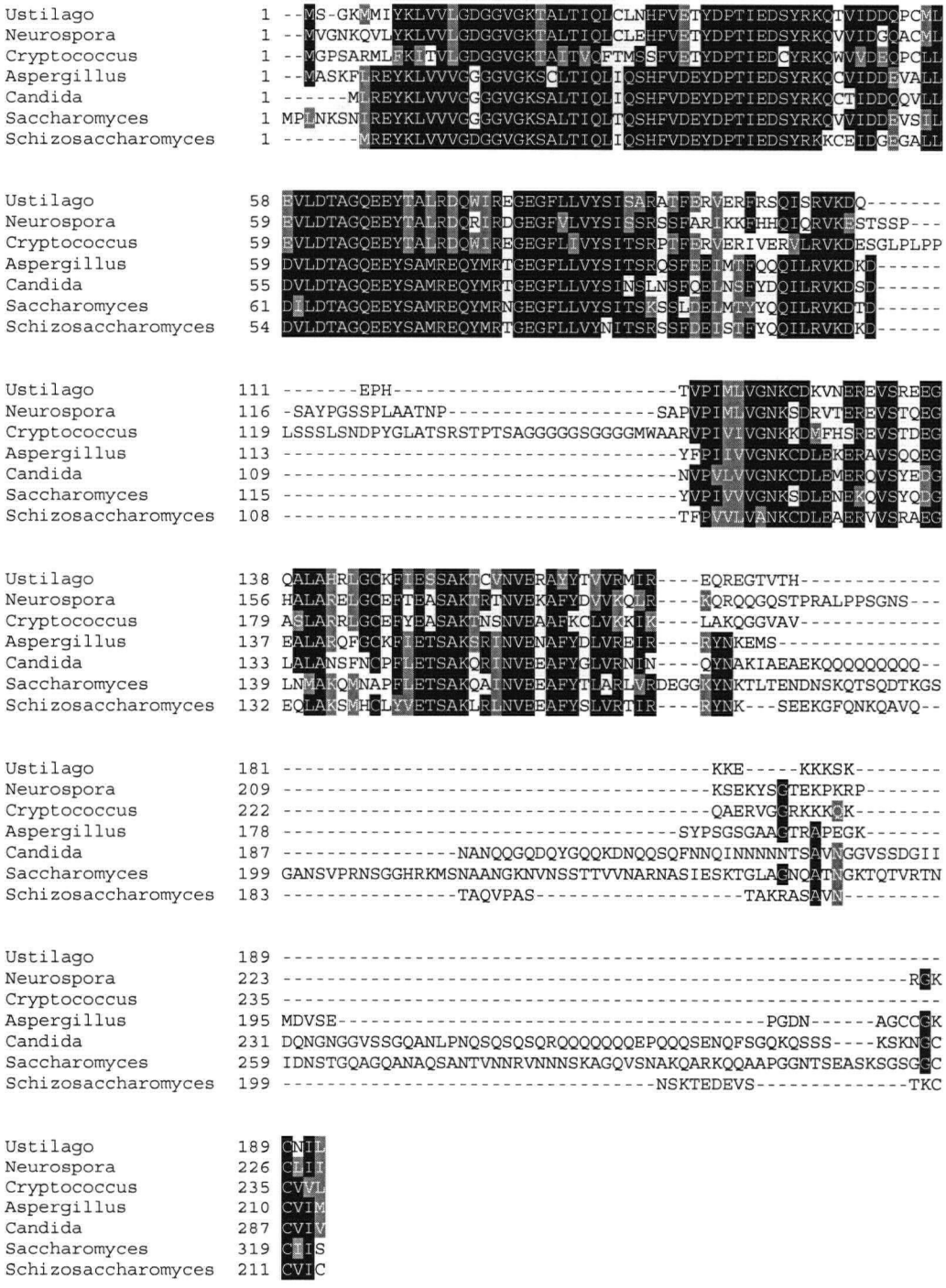


Figure 4.4. Sequence alignment of Ras proteins from other fungi with Ras1 from *U. maydis*. Identical residues are indicated by inverse print and similar amino acids are highlighted with a grey background. Sequence alignment was performed using ClustalW (Thompson et al 1994) and presented with Boxshade 3.21 (K. Hofmann and M. Boran). The proteins used for comparison are *Neurospora crassa* NC-Ras2, *Cryptococcus neoformans* Ras2, *Aspergillus fumigatus* Ras, *Candida albicans* Ras1, *Saccharomyces cerevisiae* Ras2 and *Schizosaccharomyces pombe* Ras1. The nucleotide sequence of the *ras1* gene has been submitted to the GenBank database under accession number AF545586.

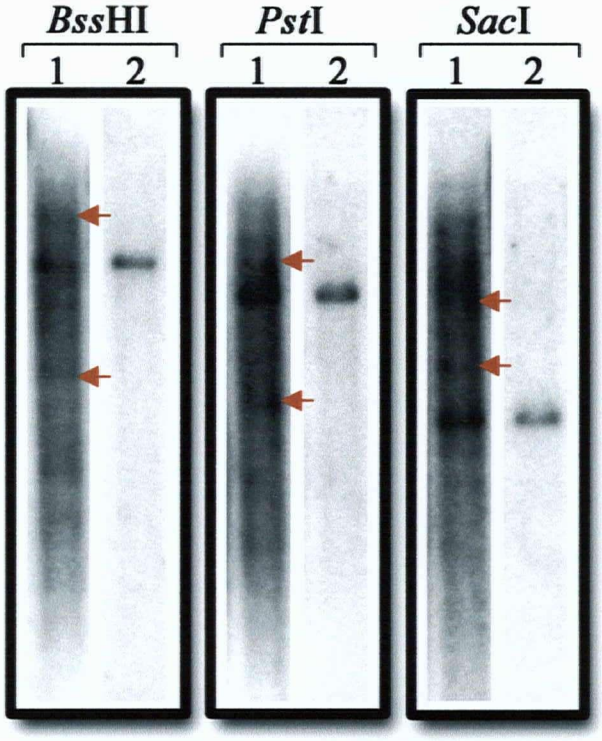


Figure 4.5 Identification of sequences with similarity to the *ras1* gene of *U. maydis*. *U. maydis* total genomic DNA gel blot hybridized with the *ras1* gene and under low stringency conditions (lane 1; Chapter 2.4.3) and under more stringent conditions (lane 2). The DNA was digested with the enzymes *Bss*HI, *Pst*I and *Sac*I as indicated. Bands representing putative sequences with high homology to *ras1* are indicated by red arrows.

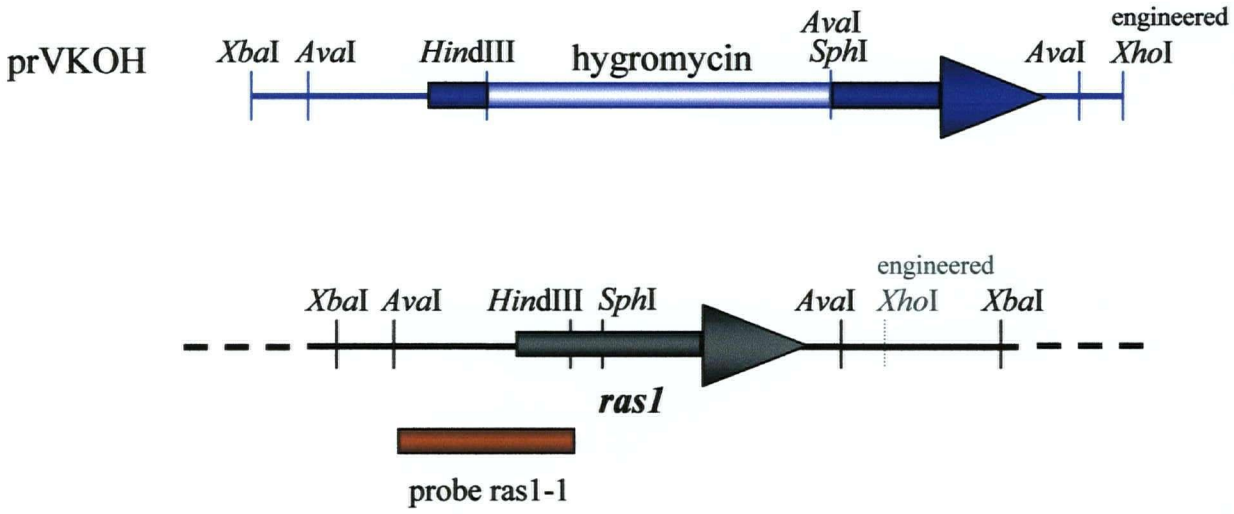
strain 33-1 because the diversity in phenotypes exhibited by the transformants suggested the possibility of copy number suppression rather than true complementation. The *ras1* allele from 33-1 was cloned by PCR and 3 independent products were sequenced; surprisingly, no mutations were found in the open reading frame of this gene (see Chapter 2.4.3). It was also unlikely that the *ras1* gene carried a mutation in the promoter region that reduced transcription levels because RNA blot analysis from mutant 33-1 and wild-type cells revealed similar levels of the *ras1* transcript (data not shown). These results suggest that the *ras1* allele found on cosmid 33-4 enables filamentous growth in the yeast-like mutant 33-1 through copy number suppression. Although the nature of the mutated gene in 33-1 remains unknown, the ability of a cosmid carrying *ras1* to complement 33-1 demonstrates that Ras1 is an important factor in morphogenesis. Thus, *ras1* is sufficient to promote filamentous growth upon transformation into the yeast-like suppressor mutant 33-1.

4.2.5 Phenotype of *ras1* deletion strains

4.2.5.1 Disruption of the *ras1* gene alters cell morphology

The *ras1* gene was deleted from each of two mating compatible haploid strains (518 and 521) to further examine its role in morphogenesis (Figure 4.6; Chapter 2.4.4). The replacement of the wild-type *ras1* allele from each strain was confirmed by hybridization analysis (Figure 4.6). Mutants lacking *ras1* were shorter and rounder than wild-type cells (Figure 4.7), and exhibited a morphology reminiscent of both *ukc1* mutants (Dürrenberger and Kronstad, 1999) and the chlamydospores described by Kusch and Schauz (1989). The *ukc1* gene encodes a protein kinase with similarity to the *cot-1* product of *N. crassa* (Yarden *et al.*, 1992). Transformation of the wild-type *ras1* allele (but not the empty vector) into *ras1* mutants restored normal cell morphology demonstrating that the phenotype observed was indeed due to deletion of the *ras1* gene (Figure 4.7). The *ras1* gene was also deleted from cells of the P6D strain. This

A



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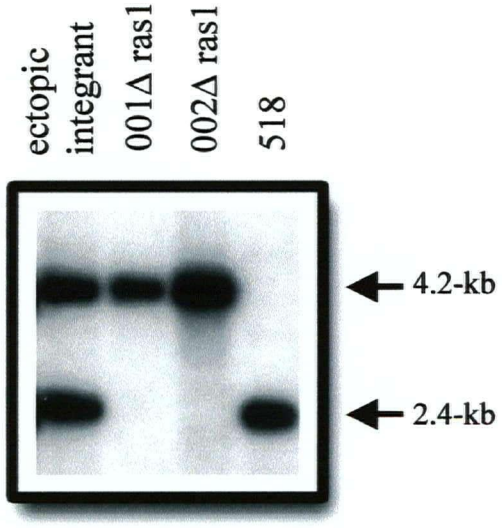

Figure 4.6 Construction and verification of a *ras1* deletion allele used to replace the wild-type *ras1* allele. Restriction enzyme maps of the wild-type *ras1* locus and the deletion construct prVKOH (A). The black line represents genomic DNA and the grey boxed arrow denotes the direction of transcription of the *ras1* gene. The site of the engineered *Xho*I recognition sequence is shown as a dotted grey line. The insert of prVKOH is drawn in blue. The location of the probe used for hybridization is shown in red. DNA hybridization analysis of the wild-type strain 518 and three transformants (B). Genomic DNA was digested with *Ava*I and hybridized with probe ras1-1. Homologous integration of the deletion construct is indicated by the hybridization of a 4.2-kb fragment and the absence of a signal from the 2.4-kb wild-type fragment.

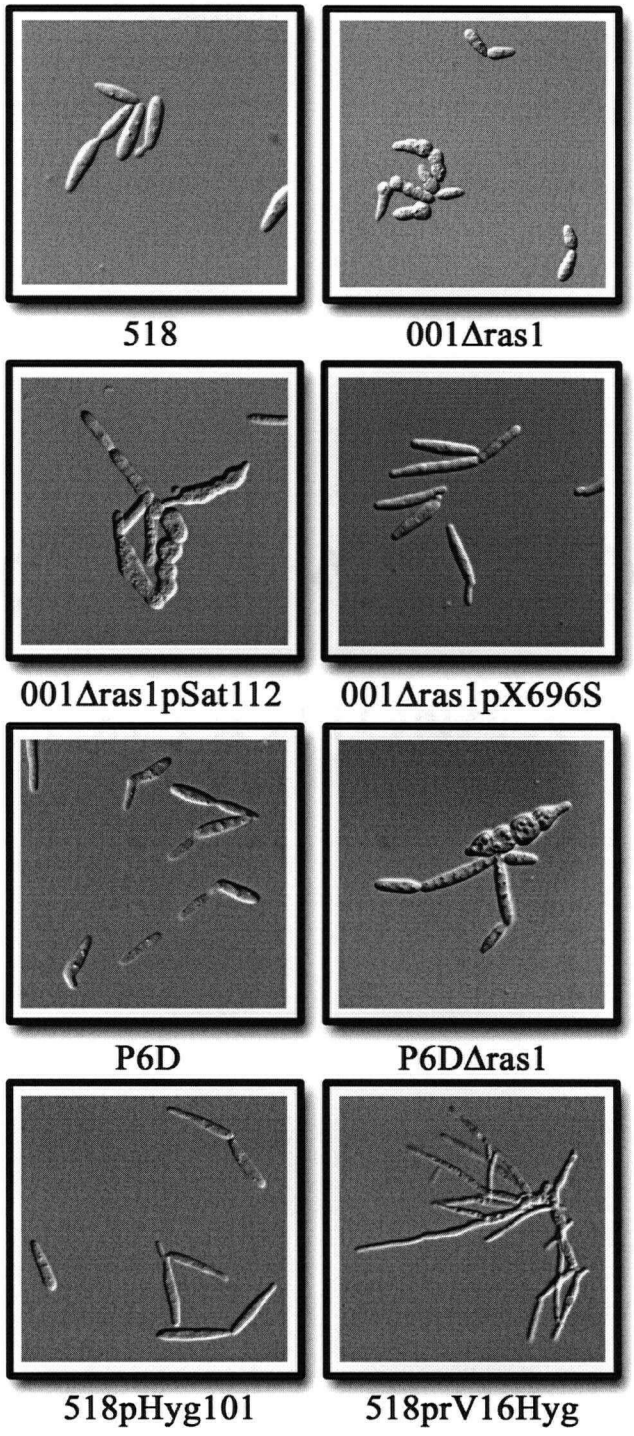

Figure 4.7. Cellular morphology of *U. maydis* strains carrying mutations at the *ras1* locus. Wild-type 518 (top left), 001Δras1 (top right), 001Δras1 transformed with a vector control (pSat112, second from top left), 001Δras1 transformed with a plasmid carrying the wild-type *ras1* allele (pX696S, second from top right), P6D (third from top left), P6DΔras1 (third from top right), wild-type 518 transformed with a vector control (pHyg101, bottom left) and wild-type 518 transformed with a plasmid carrying the activated $ras1^{Val16}$ allele (prV16Hyg, bottom right).

strain carries the *a1* and *b1* mating-type sequences randomly integrated into the genome of an *a2 b2* haploid to construct a pathogenic haploid strain due to activated mating functions (Giasson and Kronstad, 1995). The P6DΔras1 mutant displayed a rounded cell morphology similar to wild-type cells deficient of *ras1* (Figure 4.7).

It was also of interest to determine whether loss of Ras1 by deletion restored budding growth to an *adr1* mutant as expected from our original suppression screens. Repeated attempts to disrupt *ras1* in an *adr1* mutant background or *adr1* in a *ras1* mutant background were unsuccessful suggesting that this combination is lethal. To explore this possibility in more detail, we exploited the fact that transformation of wild-type cells with an *adr1* disruption construct results in a high frequency of filamentous transformants (Dürrenberger *et al.*, 1998). For example, in a screen of 200 such transformants, 43% were filamentous and hybridization confirmed *adr1* disruption in a sample (10) of these strains. By contrast, a screen of 200 transformants of a *ras1* deletion strain with the *adr1* disruption construct did not identify any filamentous strains. PCR analysis with two different primer sets confirmed that disruption of *adr1* had not occurred in these strains. Overall, these results suggest that disruption of both genes results in lethality.

4.2.5.2 Ras1 promotes filamentous growth

We constructed an activated *ras1* allele ($ras1^{Val16}$) by replacing the codon for glycine with that of valine at the 16th amino acid position to further investigate the role that Ras1 plays in morphogenesis. This dominant activating mutation is analogous to that of the $ras2^{Val19}$ allele of *S. cerevisiae* (the intrinsic GTPase activity is defective). We cloned the *U. maydis* activated $ras1^{Val16}$ allele into transformation vectors containing an autonomously replicating sequence and markers for resistance to the antibiotics hygromycin B or nourseothricin (see Figure 2.3) and

introduced these plasmids into various strains. Wild-type strains carrying these plasmids appeared yeast-like on solid medium, but these strains were clearly pseudohyphal when grown in liquid broth (Figure 4.7). As expected, wild-type strains carrying vector controls grew by budding. Interestingly, transformants of strain 33-1 with the *ras1*^{Val16} activated allele were more filamentous than those carrying the wild-type allele, while those carrying a disrupted allele (pX696rh) or the control plasmid (pHyg101; Gold *et al.*, 1994b) remained yeast-like (Figure 4.3). These results demonstrate that Ras1 acts to promote filamentous growth.

4.2.5.3 Ras1 is required for pheromone production and perception

To determine the effect of the *ras1* deletion on mating, *ras1* mutants were co-spotted either with compatible wild-type strains or as compatible mutants onto mating medium and assayed for the production of dikaryotic hyphae. Vigorous aerial hyphae were produced when *ras1* mutants were co-spotted with wild-type cells, indicating a positive mating reaction (Figure 4.8). These mating reactions were comparable to those seen when compatible wild-type cells were mated. Interestingly, *ras1* mutants were unable to induce aerial hyphae formation when co-spotted with compatible *ras1* strains, indicating that these mutants were defective in cell fusion and/or filamentous growth after fusion (Figure 4.8).

ras1 mutants were also plated next to compatible wild-type or *ras1* mutant cells to assay for the ability of *ras1* mutants to produce and respond to pheromone. Closer inspection of the mating interaction showed that *ras1* mutants were able to respond to pheromone from wild-type cells by producing conjugation tubes (Figure 4.9). However, the response to pheromone exhibited by *ras1* mutants was severely reduced in comparison to that of compatible wild-type cells plated next to each other. Furthermore, wild-type cells produced fewer conjugation tubes and responded less vigorously to *ras1* mutants, presumably because of reduced or delayed

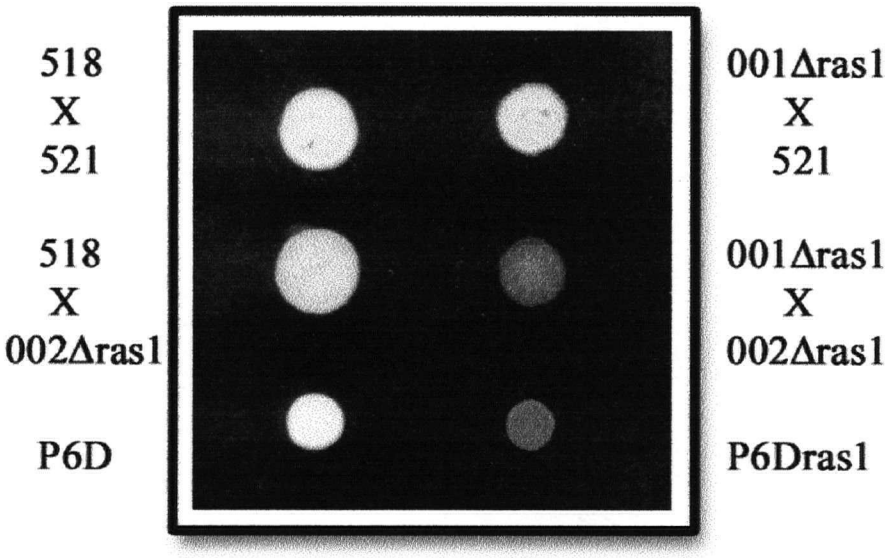

Figure 4.8. Mutants deficient of *ras1* are unable to form aerial hyphae. A strong mating reaction was seen when compatible wild-type strains were co-spotted on charcoal containing media (top left). A strong mating reaction was also observed when wild-type cells were co-inoculated with *ras1* mutants (top right and middle left). Co-inoculation of compatible *ras1* mutants resulted in a yeast-like colony (middle right). P6D cells are capable of producing aerial hyphae when inoculated without a mating partner (bottom left), but P6D cells defective in *ras1* are not able to produce these hyphae (bottom right).

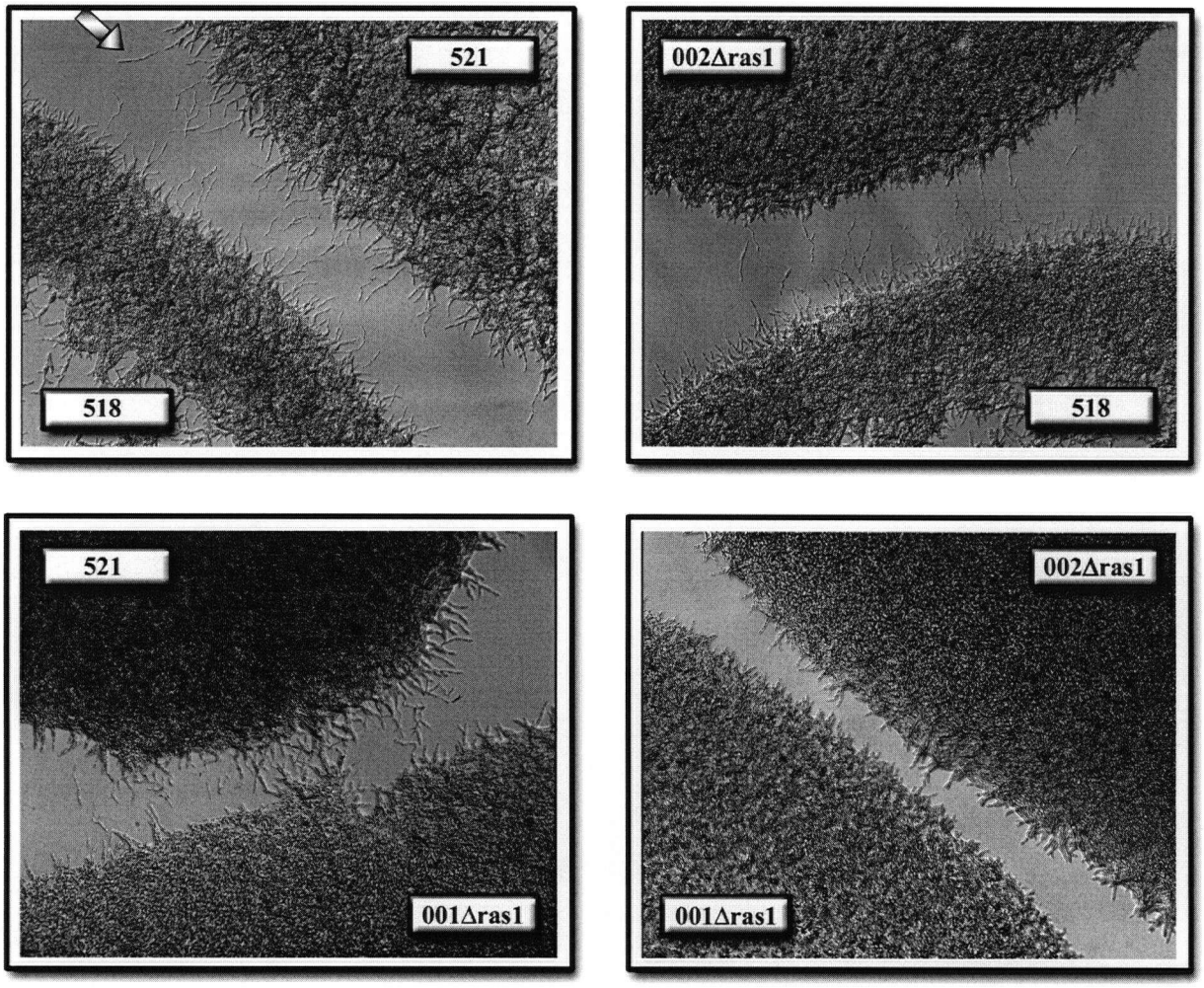

Figure 4.9. A confrontation assay indicates that *ras1* mutants produce less pheromone and are attenuated for pheromone signaling. Wild-type cells respond to pheromone from compatible cells by producing conjugation tubes (indicated by arrow) that are oriented towards their mating partner (top left). Mutants deficient in *ras1* produce very few conjugation tubes when spotted next to wild-type cells. Conversely, fewer conjugation tubes are formed by wild-type cells in response to pheromone produced from *ras1* mutants (top right and bottom left). *ras1* mutants fail to produce conjugation tubes when spotted beside compatible *ras1* partners (bottom right).

pheromone secretion. Even when compatible *ras1* mutants were spotted in very close proximity to each other, there was a complete lack of conjugation tube formation (Figure 4.9). These results indicate that *ras1* mutants are attenuated for pheromone response and suggest that they produce less pheromone than wild-type cells.

To further investigate pheromone signaling, total RNA from wild-type and *ras1* mutant cells was isolated and examined for the amount of *mfa1* pheromone gene transcript produced in each of the strains. Previous experiments have shown that a basal level of mating pheromone is expressed in wild-type cells (Urban *et al.*, 1996a). Similarly, hybridization with the *mfa1* gene demonstrated that the *mfa1* transcript was produced in wild-type cells carrying the control vector pHyg101 (Figure 4.10). Interestingly, expression of the *mfa1* gene was dramatically increased in wild-type cells carrying a plasmid containing the $ras1^{Val16}$ allele, while *mfa1* expression was completely abrogated in *ras1* mutants. These results show that Ras1 is necessary for signaling events leading to the production of pheromone in *U. maydis*.

4.2.5.4 Ras1 is essential for post-fusion filament formation and pathogenicity

The *ras1* mutant was co-inoculated with wild-type cells or compatible *ras1* mutant cells into maize seedlings to ascertain whether the *ras1* gene plays a role in pathogenicity. Similar to the results obtained from the mating assays, *ras1* mutants were pathogenic on maize when paired with wild-type cells, as expected from the positive mating reaction between these strains (Table 4.4). However, compatible *ras1* mutants were unable to induce disease symptoms, even four weeks after inoculation thus indicating that *ras1* is required for the induction of disease symptoms on maize.

The P6DΔras1 deletion mutant was used to determine whether the defects in mating and pathogenicity of haploid *ras1* mutants were due to a defect in cell fusion. The P6D strain is solopathogenic because it can form aerial hyphae on charcoal plates and induce disease

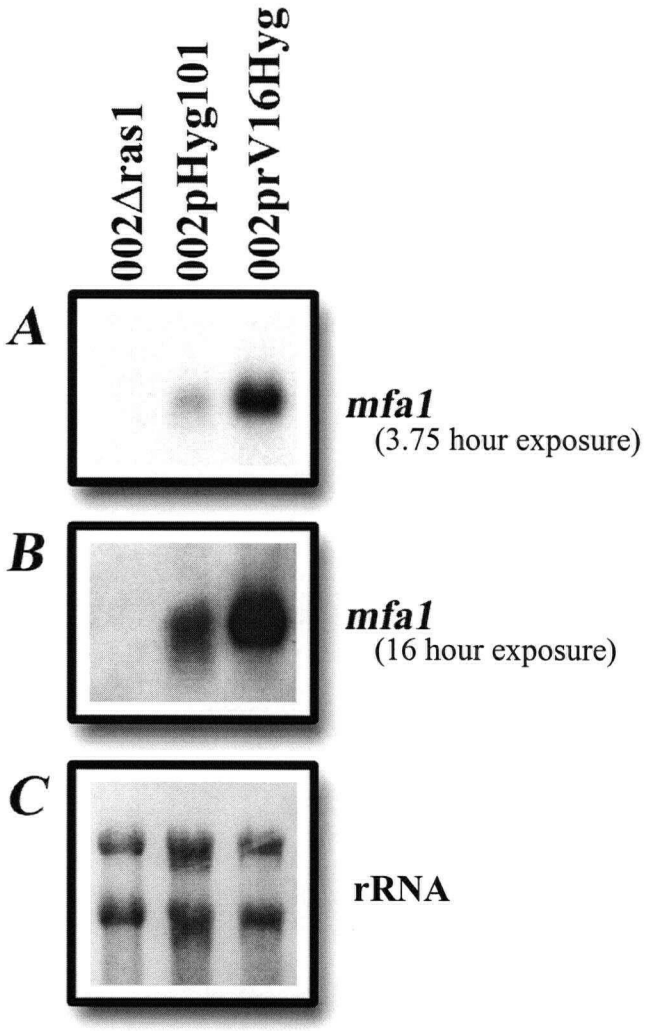

Figure 4.10. *mfa1* transcript levels in *ras1* mutants. Total RNA was isolated from 002Δras1 cells, wild-type 521 cells carrying the control vector pHyg101 (strain 002pHyg101) and wild-type 521 cells carrying the activated $ras1^{Val16}$ allele in prV16Hyg (strain 002prV16Hyg). The RNA blot was hybridized with a probe for the *mfa1* gene and exposed for 3.75 hours (A) or 16 hours (B) or stained with 0.04% methylene blue (C).

symptoms in maize seedlings in the absence of a mating partner. Deletion of the *ras1* gene in the P6D background resulted in cells that were unable to form aerial filaments on mating media (Figure 4.8). Even though P6D is weakly pathogenic, deletion of the *ras1* gene in this background further attenuated symptom formation and resulted in the complete loss of anthocyanin production and tumor formation upon injection into maize seedlings (Table 4.4). Interestingly, P6D cells carrying the activated *ras1*^{Val16} allele appeared to be more virulent in maize seedlings compared with cells carrying the vector control (Table 4.4). Multiple tumors were observed around the site of infection in seedlings infected with the P6D*ras1*^{Val16} mutant, whereas only single small tumors were seen when the untransformed P6D strain was used as inoculum (Figure 4.11). These results indicate that Ras1 plays an essential role in post-fusion events involved in filament formation and pathogenicity. Given the influence of the *ras1* gene on pheromone gene transcription, it is likely that the gene also is required for fusion during mating.

4.2.6 Ras1 and PKA regulate morphogenesis in distinct pathways

The ability of an activated *ras1* allele to promote filamentation prompted an investigation into the relationship between Ras1 and pathways known to regulate filamentous growth in *U. maydis*. One of the factors regulating the switch between budding and filamentous growth is the activity level of PKA; mutants with low PKA activity grow filamentously while mutants deficient of the regulatory subunit of PKA (encoded by *ubc1*) have a multiple budding phenotype. To examine the interactions between Ras1 and cAMP signaling, we introduced perturbations in *ras1* signaling into strains deficient in components in the cAMP pathway. To this end, the activated *ras1*^{Val16} allele was transformed into the constitutively budding *ubc1* mutant. Interestingly, *ubc1ras1*^{Val16} double mutants displayed a combination of the *ubc1* and *ras1*^{Val16} phenotypes; multiple buds were formed at the tips of elongated cells (Figure 4.12). The

Table 4.4 Pathogenicity assays with *ras1* mutants

Genotype of Strains Crossed	Total Plants Inoculated	Plants with Anthocyanin Production	Plants with Tumor Induction	% Plants with Tumours
a1b1 X *a2b2*	26	23	23	96%
a1b1 X *a2b2ras1*	51	51	51	100%
a1b1ras1 X *a2b2*	48	47	47	98%
a1b1ras1 X *a2b2ras1*	81	0	0	0%
a2b2mfa1bE1 (P6D)	47	39	8	17%
a2b2mfa1bE1ras1 (P6DΔras1)	133	0	0	0%
a2b2mfa1bE1ras1^{Val16} (P6D*ras1*^{Val16})	53	45	40	76%

These results are representative of four independent experiments.

Figure 4.11. Ras1 promotes tumor formation in a weakly virulent strain. Anthocyanin production and the formation of very small tumors are the major symptom of disease in maize seedlings infected with the P6D strain (top), while multiple tumors of varying sizes are induced upon infection with the P6D strain carrying the activated *ras1*^{Val16} allele (in prV16Hyg, bottom).

appearance of this novel phenotype suggests that Ras1 and Ubc1 may act in different pathways to regulate morphogenesis. The *hgl1* gene was recently identified as an additional component of the cAMP pathway (Dürrenberger *et al.*, 2001). The product of this gene may serve as a target for PKA and function to suppress budding growth, as *hgl1* mutants have a constitutively budding phenotype (Dürrenberger *et al.*, 2001). The introduction of the activated $ras1^{Val16}$ allele into an *hgl1* mutant resulted in filamentous transformants, in marked contrast to the budding phenotype of *hgl1* mutants transformed with the vector control (Figure 4.12). These results illustrate that budding growth resulting from a defect in *hgl1* can be bypassed by the activation of filamentous growth as a result of Ras1 activity. Overall, these results suggest that the Ras1 and cAMP pathways act antagonistically to control morphogenesis in *U. maydis*.

4.2.7 Ras1 regulates morphogenesis via a MAP kinase signaling cascade

To determine the role that Ras1 plays in filamentous growth in relation to the MAPK/pheromone response cascade, strains deficient in components of the pheromone signaling pathway were transformed with the $ras1^{Val16}$ activated allele. The *fuz7* and *ubc3* genes encode a MAP kinase kinase and a MAP kinase, respectively, and mutations in these genes suppress the constitutively filamentous phenotype of a mutant lacking adenylyl cyclase (Mayorga and Gold, 1999). Strains deficient for *fuz7* or *ubc3* however, maintain a wild-type cellular morphology (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999; Muller *et al.*, 1999). Thus, we were interested in determining the phenotype of *fuz7* and *ubc3* mutants expressing the activated $ras1^{Val16}$ allele. Considering the involvement of Ras1 in pheromone signaling, it was not surprising that the addition of the $ras1^{Val16}$ allele to *fuz7* or *ubc3* mutants resulted in strains that were no different from those transformed with the vector control (Figure 4.12; Banuett and Herskowitz, 1994). The *prf1* gene encodes a transcription factor required for pheromone response (Hartmann *et al.*, 1996). The introduction of the activated $ras1^{Val16}$ allele

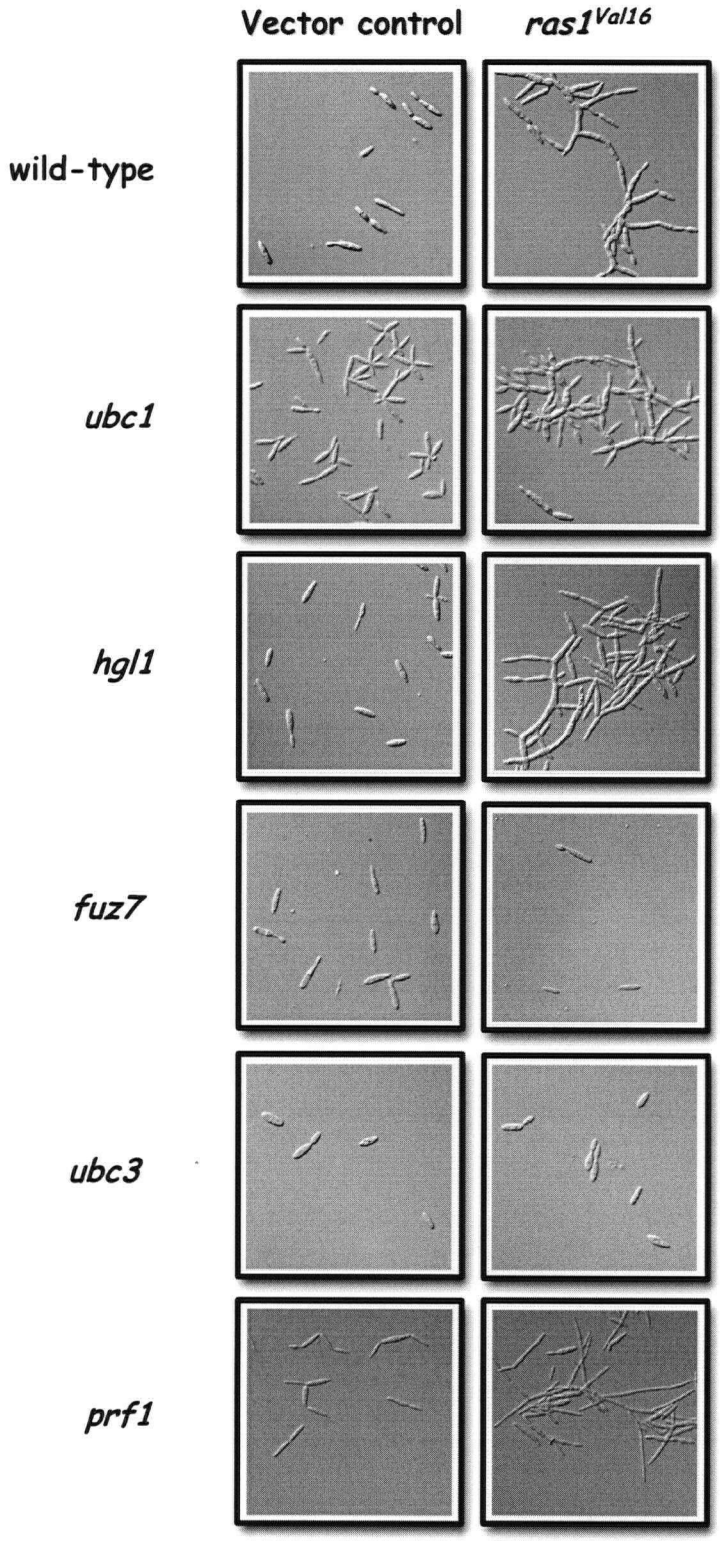

Figure 4.12. Cellular phenotype of mutants with defects in Ras1 and components of the cAMP or MAPK signaling pathways. Wild-type 518, *ubc1*, *hgl1* and *ubc3* cells were transformed with the vector control pHyg101 (left column) or a plasmid containing the activated *ras1*^{Val16} allele, prV16Hyg, (right column). *fuz7* and *prf1* mutant cells were transformed with a vector control pSat112 (left column) or a plasmid containing the activated *ras1*^{Val16} allele, prV16Sat, (right column).

into the *prf1* mutant strain however, resulted in cells with a filamentous cell morphology. As expected, transformation of the empty vector control did not influence the yeast-like cell morphology of the *prf1* strain (Figure 4.12; Hartmann *et al.*, 1996). These results indicate that Ras1 may regulate morphogenesis by signaling via a MAP kinase cascade that includes components encoded by the *fuz7* and *ubc3* genes, but not the transcription factor encoded by *prf1*. It is likely that a different transcription factor influences filamentous growth in response to signaling from Fuz7 and Ubc3.

4.3 Discussion

Ras proteins are important components of signaling cascades in many organisms and act as molecular switches by alternating between GDP and GTP bound forms in response to environmental stimuli. The involvement of Ras proteins in fungal cell growth and differentiation has been well documented. For example, in *Saccharomyces cerevisiae*, an increase in Ras2 activity is correlated with sensitivity to environmental stress, growth defects on carbon sources other than glucose, the loss of carbohydrate reserves, a transient arrest in G1, a block in sporulation, and enhanced pseudohyphal growth. *Candida albicans* mutants deficient in both copies of the *RAS1* gene exhibit defects in filament formation and virulence (Feng *et al.*, 1999; Leberer *et al.*, 2001). The *NC-ras2* gene of *Neurospora crassa* regulates hyphal growth, cell wall synthesis and conidial formation (Kana-uchi *et al.*, 1997). In *Cryptococcus neoformans*, *RAS1* is required for growth at elevated temperatures, mating, filamentation, agar invasion and sporulation (Alspaugh *et al.*, 2000; Tanaka *et al.*, 1999). The *Schizosaccaromyces pombe ras1* gene is involved in pheromone response, morphogenesis and sporulation (Fukui *et al.*, 1986; Xu *et al.*, 1994). Given these observations, it is not surprising that the Ras ortholog encoded by *ras1* in *U. maydis* is also necessary for several processes including morphogenesis, mating and virulence.

Appropriate regulation of PKA activity has been shown to be crucial for dimorphism and pathogenicity in *U. maydis* (Dürrenberger *et al.*, 1998; Gold *et al.*, 1994a; Gold *et al.*, 1997; Kruger *et al.*, 2000). In this report, we used a constitutively filamentous PKA mutant in a genetic screen to identify downstream factors of cAMP signaling and we discovered *ras1*, a member of the Ras family of small GTP-binding proteins. Haploid wild-type and P6D cells deficient in *ras1* had an altered cellular morphology, were unable to form aerial hyphae on mating medium and were severely compromised for virulence. The findings that Ras1 activation in wild-type, *ubc1*, *hgl1* or *prf1* cells resulted in pseudohyphal growth, but had no effect on *fuz7* and *ubc3* mutants demonstrates that Ras1 mediates filamentous growth via a MAPK pathway that does not impinge on the cAMP signaling pathway nor the pheromone response specific transcription factor Prf1. The involvement of Ras1 in mating was shown by the failure of compatible *ras1* mutant strains to form conjugation tubes in confrontation assays and mate on charcoal containing media. In addition, RNA blot analysis revealed that Ras1 controls pheromone gene expression. A third process requiring Ras1 signaling was discovered after maize seedlings inoculated with compatible *ras1* mutants remained completely asymptomatic. The inability of the P6DΔras1 mutant to induce disease symptoms added further evidence of the direct role of Ras1 in pathogenicity, and demonstrated that pheromone signaling and cell fusion were unlikely to be the sole causes for a loss in virulence in strains deficient of the *ras1* gene. Thus, signaling via Ras1 is essential for at least three different processes in *U. maydis*: mating, morphogenesis and pathogenesis.

4.3.1 The Ras1 and PKA pathways have opposing effects on morphogenesis

Ras proteins are bound to GTP in their active state and then become inactive upon GTP hydrolysis to GDP. In *S. cerevisiae*, the intrinsic GTPase activity of Ras2p was reduced by specifically altering glycine 19 to eliminate GTPase activity (Kataoka *et al.*, 1984; Toda *et al.*,

1985). We constructed a similar dominant *U. maydis ras1* allele by substituting glycine at the equivalent position (Gly^{16}) with valine. Introduction of this $ras1^{Val16}$ activated allele into wild-type cells resulted in transformants with a filamentous cell morphology (Figure 4.7). These cells differed from the normal unipolar, budding wild-type cells in that they were elongated, defective in cytokinesis and had multiple daughter cells growing from both ends of the mother cell.

The multiple-budding phenotype was first observed in mutants with constitutively active PKA due to a defect in the *ubc1* gene (Figure 4.12; Gold *et al.*, 1994a). The phenotype of *ubc1* mutants resembles that of activated $ras1^{Val16}$ mutants at first glance, however several lines of evidence indicate that the PKA and Ras1 pathways mediate distinct processes. While *ubc1* mutants are most often observed as small clusters of cells joined at a single tip, $ras1^{Val16}$ mutants can be isolated as large clumps. In addition to the elongated cell size of $ras1^{Val16}$ mutants, their bipolar growth pattern may account for the distinction between *ubc1* and $ras1^{Val16}$ phenotypes. *ubc1* mutants carrying the $ras1^{Val16}$ allele display a unique phenotype: bipolar multiple budding cells that are somewhat swollen, yet still elongated. Thus, it appears that the activation of PKA may serve to promote budding growth, or repress filamentous growth, by the initiation of bud sites, while the Ras1 pathway may act to promote filamentous growth through cell elongation and the inhibition of cell separation (Figure 4.13). A similar separation of morphological control by different pathways has been described for *S. cerevisiae*. Pseudohyphal growth in yeast involves cell elongation, unipolar budding, mother-daughter cell adhesion and invasive growth. The PKA pathway is thought to regulate unipolar budding and agar invasion because *tpk2/tpk2* mutants are defective for agar invasion and unipolar cell division, and strains mutated at the *ste12* loci are incapable of agar invasion and growing with an elongated cell morphology. The MAP kinase cascade regulates cell elongation and invasion (Liu *et al.*, 1993; Mosch *et al.*, 1996; Pan and Heitman, 1999; Roberts and Fink, 1994; Roberts *et al.*, 1997).

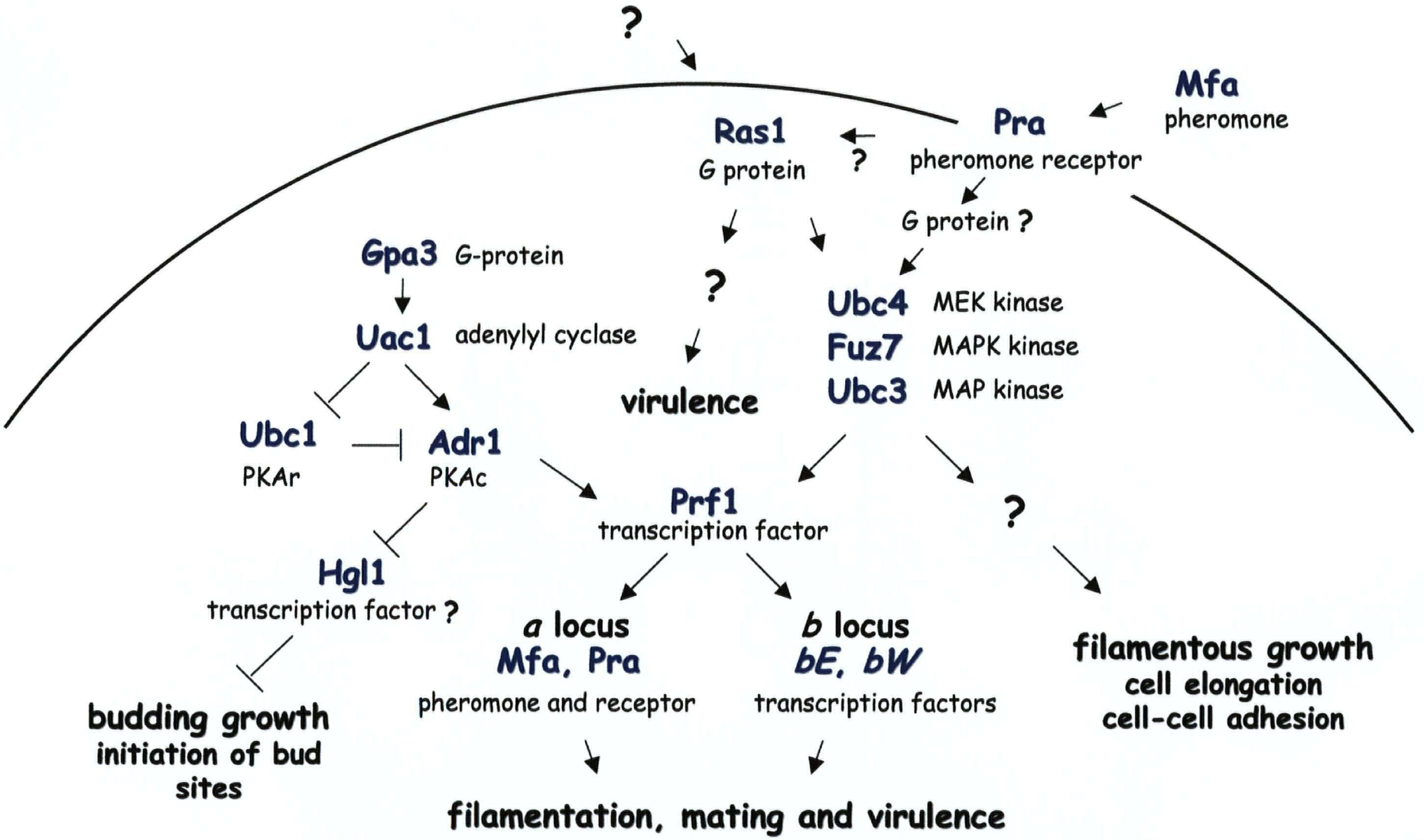

Figure 4.13 Model of the pathways regulated by Ras1 in *Ustilago maydis*. Note that PKAr refers to the regulatory subunit and PKAc is the catalytic subunit of PKA.

Although the pathway regulated by PKA may appear to counter the Ras1 pathway, the processes that they regulate may not be completely disparate because a defect in cytokinesis is associated with the activation of both pathways. Interestingly, the phenotype of wild-type cells carrying the activated *ras1*^{Val16} allele mutants is very similar to that of *uac1ubc1*, *uac1ubc2*, *uac1ubc3*, *uac1ubc4* and *uac1fuz7* double mutants (Andrews *et al.*, 2000; Gold *et al.*, 1994a; Mayorga and Gold, 1999; Mayorga and Gold, 2001). For example, the *uac1ubc1* double mutant appears to be slightly filamentous due to an elongated cell morphology (Gold *et al.*, 1994a). This indicates that adenylyl cyclase may not only produce cAMP to activate PKA but may also play additional PKA independent roles in morphogenesis. Whether these supplementary roles are associated with Ras1 activity remains to be determined.

4.3.2 The Ras1 pathway regulates filamentation through a MAP kinase pathway

The activation of Ras1 failed to induce filamentous growth in mutants deficient in the MAPK Ubc3 or the MAPKK Fuz7, indicating that Ubc3 and Fuz7 constitute part of a MAP kinase cascade that relays signals from Ras1 to influence cell elongation and cytokinesis (Figure 4.13). The genetic interaction between *ras1*, *ubc3* and *fuz7* is consistent with the fact that both *ubc3* and *fuz7* were identified based on their ability to complement secondary mutations that suppressed the constitutively filamentous phenotype of mutants deficient in the *uac1* gene (Andrews *et al.*, 2000; Mayorga and Gold, 1999).

Filamentous growth resulting from the activation of Ras proteins has been observed in a number of yeasts and fungi. In response to nitrogen starvation, diploid *S. cerevisiae* cells undergo pseudohyphal growth, which is enhanced by the expression of the dominant-active allele of *RAS2* (Gimeno *et al.*, 1992). Further investigation revealed that pseudohyphal growth is caused by the activation of a MAP kinase pathway by *RAS2* (Mosch *et al.*, 1996; Roberts *et al.*, 1997). Similarly, *C. albicans* strains carrying the activated *RAS1*^{V13} allele formed more abundant

hyphae in a shorter time period than wild-type strains (Feng *et al.*, 1999). Under conditions of nitrogen starvation and in response to mating pheromone, certain strains of *C. neoformans* are capable of forming filaments and sporulating in the absence of a mating partner (Wang *et al.*, 2000; Wickes *et al.*, 1996). This process, known as haploid fruiting, does not normally occur in the serotype A strain H99, however, vigorous haploid fruiting was observed in H99 cells expressing the activated $RAS1^{Q67L}$ allele (Alspaugh *et al.*, 2000). The Gβ subunit encoded by *GPB1* was also determined to be involved in the regulation of haploid fruiting (Wang *et al.*, 2000). Gpb1 was implicated as a downstream component of the Ras1 signaling pathway because expression of the activated $RAS1^{Q67L}$ allele in *gpb1* mutants failed to induce haploid fruiting in the H99 strain. Furthermore, Gpb1 signals through a MAP kinase cascade to regulate filamentous growth in response to mating (Wang *et al.*, 2000).

It is generally thought that pseudohyphal growth in *S. cerevisiae* enables this normally nonmotile organism to forage for nutrients under adverse conditions. In an analogous manner, the pseudohyphal cell type in *U. maydis* may represent a nutritionally stressed growth form that develops when mating between haploid cells occurs away from host tissue. Because the dikaryotic cell type requires the host environment to proliferate, the unsuccessful mating partners may revert to pseudohyphal growth to search for more appropriate surroundings. Pseudohyphal growth may then be repressed when conditions are favourable.

The filaments formed after the activation of Ras1 are distinct from those produced because of low PKA activity (i.e. loss of Uac1 or Adr1), as well as those formed in response to mating (i.e. conjugation tubes) and those formed after cell fusion (i.e. dikaryotic filaments) for that matter. While conjugation tubes have been described as long, thin and often coiled filaments that extend from one polar end of a cell, (Snetselaar, 1993; Snetselaar and Mims, 1992) and dikaryotic filaments are viable only *in planta* and are composed of 1) a combination of cells containing two nuclei and seemingly empty or "vacuolated" cells, 2) branched filaments, and 3)

branch primordia that resemble clamp connections, (Banuett and Herskowitz, 1996) mutants deleted for *uac1* or *adr1* develop as straight-growing filaments with single nucleated cells separated by septa (Barrett *et al.*, 1993). In contrast, cells expressing the activated $ras1^{Val16}$ allele were pseudohyphal, in that elongated individual cells remained attached to one another, but clearly grew by budding. The discrepancy between the cellular morphology of filamentous cells growing *in planta* and that of pseudohyphal cells expressing the activated $ras1^{Val16}$ allele may be attributed to ploidy and/or environmental factors. Our observations of pseudohyphal growth were based on haploid monokaryons that were grown under very strict conditions, whereas previous descriptions of filamentous growth by the dikaryotic cell type were made of cells growing within host tissue (Banuett and Herskowitz, 1996; Snetselaar and Mims, 1992; Snetselaar and Mims, 1993). It should be noted that differences in gene expression patterns exist between the various filamentous structures in *U. maydis* (Basse *et al.*, 2000). For example, the *mig1* gene is weakly expressed in a diploid strain grown on media containing activated charcoal, (which induces filamentous growth in this strain), highly expressed in filaments proliferating in planta and not expressed at all in hyphae growing on the leaf surface. Alternatively, filamentous growth may be triggered by a signal emanating from the host environment that activates a pathway involving Ras1, Fuz7 and Ubc3 (Figure 4.13). Future research may focus on the identification of potential host factors that elicit filamentous growth and signal through Ras1 in *U. maydis*.

4.3.3 The *ras1* gene regulates pheromone expression

Mating and dimorphism are intricately connected in *U. maydis* because haploid cells must first mate before undergoing the morphological switch to filamentous growth. Therefore, it would seem appropriate that the factors controlling these processes are coordinately regulated. In fact, many of the factors mediating pheromone response are also responsible for filamentous

growth. In this thesis, it was found that Ras1 plays a central role in both mating and dimorphism.

The lack of filament formation upon co-inoculation of compatible *ras1* mutants, the absence of pheromone expression in *ras1* mutant cells and the increased production of *mfa1* gene transcripts in cells expressing the activated $ras1^{Val16}$ allele provide evidence of the importance of Ras1 in mating. In confrontation assays between wild-type cells and *ras1* mutants the reduced vigor with which conjugation tubes were formed from wild-type cells indicates that *ras1* mutants are capable of pheromone secretion, although pheromone production may be reduced or delayed. It is possible that another G protein may either play a minor role in pheromone signaling or be able to substitute, albeit inefficiently, for the loss of Ras1, since pheromone production and conjugation tube formation were observed at reduced levels in *ras1* mutants. The detection of several bands using sequences from the *ras1* locus after hybridization under low stringency conditions indicate that additional Ras-like proteins may exist in *U. maydis* (Figure 4.5). Certainly, functional overlap between Ras proteins has been documented in *C. neoformans* and *S. cerevisiae*. For example, overexpression of the *C. neoformans RAS2* gene fully suppresses the mating defect of a *ras1* mutant and partially suppresses the *ras1* mutant morphological and high temperature growth defects (Waugh *et al.*, 2002). In a similar manner, the overexpression of the *RAS1* gene of *S. cerevisiae* restores invasive growth to *ras2* mutants (Mosch *et al.*, 1999; Powers *et al.*, 1984). Alternatively, a separate pathway may be able to respond to pheromone and activate the transcription of genes at the mating-type locus. For example, the cAMP pathway has been shown to influence pheromone signaling as *ubc1* mutants express elevated levels of *mfa1* transcript. (Kruger *et al.*, 1998) Taken together, these results show that Ras1 is required for the basal expression of mating pheromone and that a Ras1 independent pathway exists for the amplification of pheromone expression in response to pheromone from compatible cells (Figure 4.13).

Mutants deficient for components of the pheromone response pathway exhibit phenotypes that are similar to that of the *ras1* mutant. First, *ubc3* mutants fail to produce aerial hyphae when co-spotted on mating medium much like the *ras1* mutant (Mayorga and Gold, 1999). Further analysis using drop mating and RNA blot assays confirmed that *ubc3* mutants produce less pheromone and are incapable of responding to pheromone produced by compatible mating partners (Mayorga and Gold, 1999; Muller *et al.*, 1999). Secondly, haploid *fuz7* mutants show reduced filament formation during mating interactions and diploid *fuz7* mutants are yeast-like after 24 hours of growth on charcoal agar (Banuett and Herskowitz, 1994). The role that Fuz7 plays in pheromone response is somewhat unclear though. Banuett and Herskowitz (1994) found that Fuz7 is necessary for conjugation tube formation and aerial filament formation during mating. However, Regenfelder *et al.* (1997) reported opposing effects of the *fuz7* mutation on the basal pheromone expression levels depending on strain background. They also co-cultured strains differing in *a* but identical in *b* and showed that the amount of *mfa1* transcript expressed is reduced in strains lacking *fuz7*, compared to wild-type strains. Lastly, pheromone signaling through the MAP kinase cascade leads to increased transcription of the pheromone response factor, encoded by the *prf1* gene (Hartmann *et al.*, 1996; Mayorga and Gold, 1999; Muller *et al.*, 1999). The increased production of Prf1 in response to pheromone explains an observation by Urban *et al.* (1996) that a 10 to 50-fold increase in the expression of the mating-type genes occurs after pheromone stimulation. Mutants deleted for *prf1* are sterile because of an inability to produce and perceive pheromone (Hartmann *et al.*, 1996). One of the main roles of Prf1 seems to be the induction of the mating-type genes, as the constitutive expression of the *bE1* and *bW2* genes in a solopathogenic strain expressing a *prf1* deletion allele (*a1bW2bE1Δprf1*) restores filamentous growth and pathogenicity to this strain (Hartmann *et al.*, 1996). Thus, it seems likely that Ras1 signals through Fuz7, Ubc3 and Prf1 to regulate pheromone response (Figure 4.13).

4.3.4 Ras1 is a pathogenicity factor

The correlation between mating and morphogenesis can be further extended to include pathogenesis because all three processes are intricately connected in *U. maydis*. Transformation of wild-type strains with the activated *ras1*^{Val16} allele resulted in increased pheromone gene expression and an elongated cell morphology. Given that P6D cells expressing the activated *ras1*^{Val16} allele were apparently more virulent than the untransformed control, activation of the Ras1 pathway also may serve to enhance host penetration or tumor formation. In *C. neoformans*, activation of the cAMP pathway by deletion of the *PKR1* gene encoding the regulatory subunit of PKA increases virulence in both rabbit and mouse models of cryptococcosis (D'Souza *et al.*, 2001). However, there were no observable differences between maize seedlings infected with *U. maydis* wild-type cells carrying a plasmid containing the activated *ras1*^{Val16} allele or carrying an empty vector as a control. These results indicate that the increased virulence brought about by expression of the *ras1*^{Val16} allele may correct a problem specific for the P6D strain. For example, it is possible that the activation of Ras1 compensates for the weakened pathogenesis of the P6D strain by inducing the expression of virulence genes that are controlled by the pheromone response pathway. Prior to cell fusion during mating interactions, the pheromone response pathway is activated and the expression of genes regulated by this pathway is elevated. After cell fusion, the active bE/bW heterodimer represses the transcription of certain pheromone-induced genes (Urban *et al.*, 1996b). In the P6D strain, the accumulation of pheromone-induced gene products is bypassed by the presence of an active *b* gene complex, which might explain the reduced virulence of this strain. Alternatively, the activation of Ras1 may aid the P6D strain in pathogenicity simply by promoting the filamentous cell morphology. The presence of branched filaments and branch primordia in wild-type dikaryotic filaments may facilitate host tissue invasion (Banuett and Herskowitz, 1996). Thus, the multiple budding aspect of pseudohyphal

growth initiated by the activation of Ras1 may enable a single fungal filament to develop in several directions and induce multiple tumors. Although the characterization of P6D filaments *in planta* has yet to be documented, the development and morphological features of hyphae from dikaryons and diploids are indistinguishable (Banuett and Herskowitz, 1996). It is a common finding that diploid strains heterozygous at the mating-type loci are only weakly pathogenic on maize, similar to the P6D strain. It may be the case that the P6D and diploid strains do not efficiently produce filaments or other virulence traits that are necessary for aggressive proliferation in the host environment. In fact, we observed larger tumors and more obvious disease symptoms in maize seedlings infected with the d132 diploid strain carrying a plasmid containing the activated $ras1^{Val16}$ allele, compared to seedlings infected with d132 carrying an empty vector control. A third explanation for our observed results is that Ras1 may function in a pheromone independent pathway that regulates pathogenicity. Perhaps host signals are poorly perceived in diploid and P6D strains, compared to wild-type dikaryons, and these signals trigger the activation of fungal factors promoting filament proliferation, tumor induction and teliospore development through a pathway controlled by Ras1 (Figure 4.13).

The relationship between Ras1 and MAP kinase cascade components with respect to pathogenesis was not investigated here, however, the findings that mutants deleted for the *fuz7* and *ubc3* genes are still able to induce tumors and produce teliospores, (albeit at reduced levels in the case of *fuz7*), indicates that Ras1 does not act solely through a MAP kinase cascade composed of these proteins (Banuett and Herskowitz, 1994; Mayorga and Gold, 1999). It appears as though Ras1 may signal via a combination of shared and unique components depending upon the specific elicitor and process that is taking place (Figure 4.13). The use of common components in different pathways leading to different developmental fates has been best characterized in *S. cerevisiae*. Mating in response to pheromone and pseudohyphal growth in response to nitrogen limitation requires the transcription factor Ste12 and an intact MAP

kinase cascade composed of Ste20, Ste11 and Ste7. Signaling specificity is achieved by the involvement of different signaling sensors and activators of the MAP kinase cascade, different scaffolding proteins that bind the MAPK components together, different MAP kinases and different downstream transcription factors. Specifically, for filamentous growth, Ras2, Cdc42, Bmh1 and Bmh2 operate to activate the MAP kinase cascade, while pheromone, pheromone receptors and the βγ subunits of a heterotrimeric G protein lie upstream of the MAP kinase pathway during the mating process. In addition, the scaffold protein Ste5 and the MAP kinase Fus3 are required during mating, while another scaffold protein, possibly Spa2, and the MAP kinase Kss1 are implicated in filamentous growth (Madhani *et al.*, 1997). Further specificity is accomplished by the coupling of Ste12 to the Mcm1 protein during pheromone response and the combination of Ste12 and Tec1 during pseudohyphal growth.

In *U. maydis*, all of the factors associated with the MAP kinase cascade, including pheromones, pheromone receptors, Ras1, the scaffold protein Ubc2, the MAPK kinase Fuz7 and the MAP kinase Ubc3 have been implicated in both filamentous growth and pheromone response. However, the transcription factor Prf1 appears to be solely responsible for pheromone response and only Ras1 and Ubc2 are absolutely required for pathogenesis. It seems likely that Ras1 responds to multiple signals and controls different pathways that lead to the activation of diverse targets. The ability of Ras1 to discriminate between different signals and the elucidation of the downstream effectors of Ras1 will be interesting challenges for future research.

CHAPTER 5: General discussion

5.1 The identification and characterization of *Ustilago* virulence factors

One of the characteristics of smut fungi is their ability to form masses of pigmented spores that are easily dispersed to other host plants. The prolific sporulation and aggressiveness of smuts give these fungi the potential to cause devastating losses in crop yield. Much of the effort in *Ustilago* research has focused on identifying factors that are critical for host infection and survival within host tissue. However, the complexity of the plant-pathogen relationship has made it difficult to study the factors required for host-pathogen interactions directly. Furthermore, reliable primary culture systems to examine growth of the pathogenic dikaryotic hyphae are still currently unavailable, making it necessary to devise alternate approaches to investigate *Ustilago* pathogenesis. One strategy involves the characterization of factors required for processes that are more easily accessible and very tightly correlated with virulence. In the work described here, the connection between pathogenesis and two different processes, mating and morphogenesis, were used to identify and characterize virulence factors in smut fungi.

The association between mating and pathogenesis has prompted several research groups to study the components of the pheromone response pathway in *Ustilago*. A comparison of the mating-type genes in *U. hordei* and *U. maydis* revealed a high degree of homology between the genes, but a dramatically different genomic organization of the mating-type loci. Whereas the *a* and *b* gene complexes are located on two different chromosomes in *U. maydis*, the homologous genes are localized to the same chromosome in *U. hordei* (Bakkeren and Kronstad, 1994). Furthermore, preliminary studies revealed that *a* and *b* are not closely positioned on the mating-type chromosome despite their tight genetic linkage. It was then hypothesized that additional factors involved in mating and pathogenesis may be located within the unusually large *U. hordei* mating-type locus and account for the apparent suppression of recombination. By tagging the *a*

and *b* gene complexes with the recognition sequence of a rare-cutting restriction endonuclease, the size of the mating-type locus was determined to span a minimum of 500 kb.

The correlation between morphogenesis and pathogenesis has also served as a useful tool in the elucidation of factors involved in both biological events. Using a genetic screen for morphological mutants, a defined role for cAMP signaling in morphogenesis and virulence has been established (Dürrenberger *et al.*, 2001; Dürrenberger *et al.*, 1998; Gold and Kronstad, 1994). Suppressor analysis and complementation of suppressor mutants resulted in the identification of the *ras1* gene. Analysis of various *ras1* mutants revealed a role for Ras1 in mating, morphogenesis and pathogenesis.

5.2 Fungal mating-type loci, bacterial pathogenicity islands and mammalian sex chromosomes

Recent advances in whole genome analysis have facilitated the construction of a physical map of the *U. hordei* genome using BAC clone fingerprints (G. Jiang, personal communication). BAC clones containing the *a* and *b* gene complexes were identified and used to position the *MAT* locus on the largest assembled contig of the physical map (see Appendix I). A minimum tiling path of BAC clones spanning *a* and *b* was produced and sequence analysis of these clones is currently underway (G. Jiang, personal communication). Preliminary sequencing results have identified candidate genes with homology to factors known to be involved in mating, morphogenesis and pathogenesis in other fungi. In particular, genes homologous to elements of *S. cerevisiae* Ras2 mediated MAP kinase cascade are represented in the mating-type region, including *STE11* and *KSS1*. The requirement for MAP kinase signaling in pheromone response is well established and components of the mating MAP kinase cascade are known to regulate morphological responses in fungi as well (Kronstad *et al.*, 1998; Lengeler *et al.*, 2000). Thus, the *MAT* locus of *U. hordei* appears to be comprised of multiple genes involved in mating,

morphogenesis and pathogenesis. A similar organization of mating-type, signaling and virulence genes exists in the human fungal pathogen *C. neoformans* (Karos *et al.*, 2000).

In many respects, mating-type loci from bipolar basidiomycete fungi share features with both bacterial pathogenicity islands and sex chromosomes in higher eukaryotes. The *MAT* locus of *U. hordei* contains numerous genes involved in mating, virulence and possibly morphogenesis and appears to be suppressed for recombination despite the fact that it encompasses an unusually large region (Lee *et al.*, 1999). Similarly, prokaryotic pathogenicity islands carry clusters of genes involved in virulence and measure between 10 and 200 kb (Lee, 1996). They serve to increase fitness and by existing as a single genetic unit, permit the synchronous transfer of factors that give the recipient a selective advantage over non-carriers (Hacker and Carniel, 2001). Likewise with respect to sex chromosomes in higher organisms, an important feature is the suppression of recombination between the regions that control sex (Clark, 1988). It is interesting that both bacterial pathogenicity islands and mammalian Y chromosomes contain repetitive DNA elements such as transposons because initial BAC-end sequencing of the BAC clones spanning the *U. hordei MAT-1* locus revealed that approximately one fifth of the sequence reads contained elements with similarity to *S. cerevisiae* transposon Ty1 (G. Jiang, personal communication; Erlandsson *et al.*, 2000; Hacker and Carniel, 2001; Jablonka and Lamb, 1990). Further evidence linking fungal mating-type loci and higher eukaryotic sex chromosomes comes from the finding that the chromosomes carrying the mating-type loci from the smut fungus *Mycrobotryum violacum* are dimorphic (Hood, 2002). In fact, the heteromorphic nature of mammalian sex chromosomes is one of their most recognized characteristics (Jablonka and Lamb, 1990). In summary, the work on the *U. hordei* mating-type locus presents unique and fascinating insights into the integration of mating, morphogenesis and pathogenesis in fungal pathogens and the possible role of mating-type loci in the evolution of sex chromosomes.

5.3 Conserved signaling pathways regulate diverse biological processes

The *ras1* gene was identified in a screen to identify genes involved in morphogenesis in the maize pathogen *U. maydis*. The isolation of a G-protein during a screen of morphological mutants, and the discovery that Ras1 also influences virulence and pheromone response demonstrates an additional level of regulation in cell signaling and reaffirms the intricate connection between these three distinct events. MAP kinase signaling networks are responsible for cellular responses to a diverse range of stimuli. In the same manner that MAP kinase cascades mediate fungal virulence, MAP kinase signaling is an important factor in mammalian cell fate and plant defense against pathogen attacks. Mammalian MAP kinases are involved in cell proliferation and differentiation, stress response and apoptosis, while plant MAP kinase pathways are activated in response to diverse signals, such as drought, cold, wounding, touch, rain and wind (Widmann *et al.*, 1999).

5.4 Prospects for the future

The work described here sets the stage for the identification of additional genes involved in fungal pathogenesis. A list of possible follow-up experiments is presented below.

1) Characterization of genes present at the *MAT* locus of *U. hordei*.

The discovery of numerous putative virulence factors within the *U. hordei* mating-type locus has confirmed the hypothesis that formed the basis of this work. The systematic deletion of genes located within the *U. hordei MAT* locus and characterization of the corresponding mutants in mating tests, confrontation assays, RNA hybridization analysis and barley infection tests will reveal whether these genes are indeed involved in mating and pathogenesis. Given that many of these genes have homologs in *S. cerevisiae* that are linked to mating, filamentous growth and

sporulation, it is likely that the *MAT* locus of *U. hordei* does harbor several additional genes involved in the regulation of mating and pathogenesis.

2) Comparison of sequences at the *U. hordei* and *U. maydis MAT* loci.

To complement the detailed investigation of the *U. hordei* mating-type locus, sequence analysis of the *MAT-2* locus of *U. hordei* could be performed. The characterization of the *U. hordei MAT-2* locus will reveal differences between *MAT-1* and *MAT-2* and provide clues that may explain the apparent suppression of recombination within *MAT*. Furthermore, it is likely that additional genes involved in mating and pathogenesis are also encoded at *MAT-2* locus. It would be interesting to determine the degree of conservation between the *MAT-1* and *MAT-2* – encoded genes, specifically with respect to their allelic specificity, presence or absence within the locus, their location within *MAT* and the orientation of these genes in relation to the other mating-type genes.

A comparison of the sequences flanking the *a* and *b* loci in *U. maydis* and the *a* and *b* gene complexes of *U. hordei* may also reveal interesting insights into the degree of conservation between tetrapolar and bipolar fungi. Collaboration with a genomics-based drug discovery company (Exelixis, Inc.) that has sequenced the *U. maydis* genome has resulted in the acquisition of large contigs containing the *a* and *b* loci. These sequences will allow a direct comparison of the mating-type sequences from *U. hordei* and *U. maydis* and will reveal regions of similarity as well as points of divergence. A key point of interest will be to determine which sequences have been maintained in association with the *a* and *b* mating type functions and which sequences do not show a conserved association. This work may provide clues as to how the bipolar and tetrapolar mating-type systems evolved in these organisms in terms of their genome organization.

3) A screen for genetic suppressors of *ras1* mutant phenotypes.

In addition to the map-based approach described above, the continuation of a genetic strategy using a *ras1* mutant may lead to the isolation of novel virulence factors. Suppressor mutagenesis starting with the *ras1* mutant could be used to identify novel downstream effectors of Ras1-MAP kinase signaling in *U. maydis*, since the only known target of MAP kinase phosphorylation, Prf1, does not influence morphogenesis. For example, the *ras1* mutant exhibits a rounded cell morphology reminiscent of both *ukc1* mutants and the chlamydospores (Dürrenberger and Kronstad, 1999; Kusch and Schauz, 1989). Complementation of yeast-like *ras1* suppressor mutants may result in the identification of genes involved in cell polarity (encoding proteins such as actin, cell wall proteins and components of signaling cascades) and melanin formation (e.g., phenol oxidases). Furthermore, suppressors of the $ras1^{Val16}$ pseudohyphal growth phenotype could be isolated and complementation of these mutants may result in the identification of genes involved in cytokinesis, bud-site selection and cell cycle.

4) Identification of proteins that interact with Ras1.

To complement the genetic screen for genes involved in Ras1 signaling pathways, proteins that physically interact with Ras1 could also be identified by a 2-hybrid screen. Along with Ras1 specific GAP's and GEF's, Ubc2 may be shown to associate with Ras1. As mentioned above, the *ubc2* gene encodes a putative adaptor protein that may contain a putative Ras-Association domain and serve to tether the components of the MAP kinase cascade (Mayorga and Gold, 2001).

5) Whole genome and proteome approaches to identifying targets of Ras1.

To identify all of the elements affected by Ras1 signaling in *Ustilago*, genomic approaches such as transcript profiling or quantitative proteomics may be used. With information obtained

from Exelixis, Inc. and the *Ustilago* genome sequencing project initiated by the Whitehead Institute, tools such as Serial Analysis of Gene expression (SAGE), micro-array analysis and the isotope coded affinity tag (ICAT) based strategy can be used identify Ras1 targets. Comparison of the transcript and protein profile from wild-type cell and *ras1* mutants will likely reveal virulence factors present only in the wild-type cells. These virulence factors may include membrane proteins that facilitate filament formation and fungal proliferation within the host. Candidate genes should then be tested for their role in virulence by constructing knock-out mutants and assaying these mutants for their ability to induce disease symptoms in maize. The identification and characterization of Ras1 targets may result in a clearer understanding of pathogenesis in the smut fungi.

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APPENDIX I: Identification of BAC clones carrying the *a* and *b* gene complexes from *U. hordei* and a homolog of the *U. maydis hgl1* gene

Introduction

Several different cloning vectors exist for amplifying DNA in bacterial cells including plasmids, phage, cosmids and bacterial artificial chromosomes. While plasmids and phage carry relatively small fragments of foreign DNA (0.1 – 20 kb), cosmids are capable of containing inserts between 35 and 50 kb. Bacterial artificial chromosome (BAC) vectors are used to clone large DNA fragments (100- to 300-kb insert size) in *E. coli* cells. The advantages of BAC vectors are: 1) the ability to clone large inserts, 2) their stable maintenance in *E. coli* cells, and 3) the low copy number of BAC clones within a given cell (one to two copies per cell), which reduces the potential for recombination between cloned DNA fragments and avoids counter-selection due to over-expression of genes.

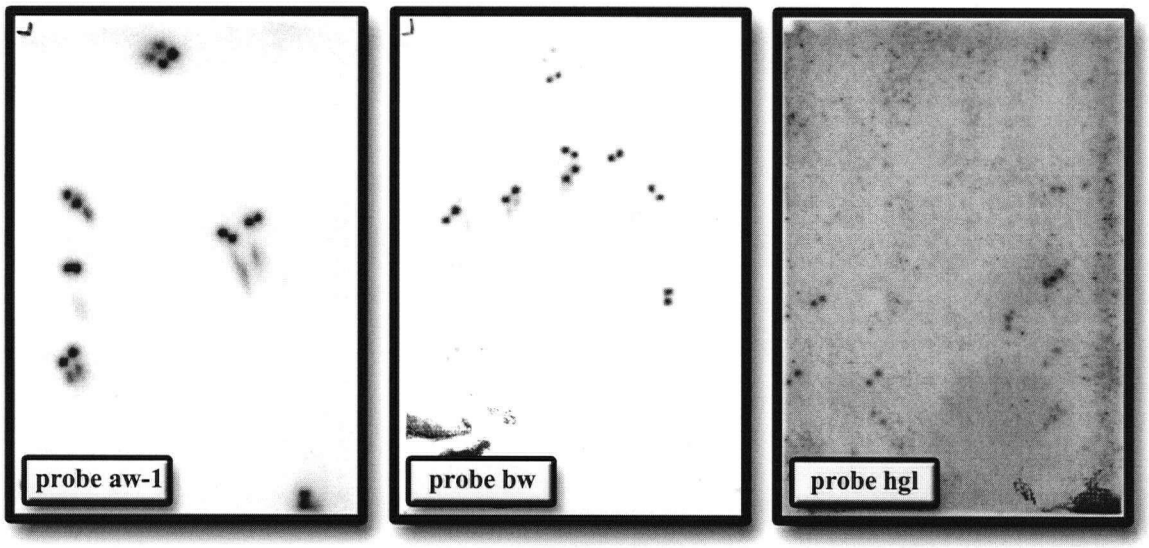
A BAC library composed of genomic DNA from *U. hordei* strain 4857-4 was supplied by Genome Systems, Inc. The availability of this library presented an opportunity to isolate BAC clones containing sequences from the *a* and *b* gene complexes of *U. hordei*, which could then be used to further characterize the *MAT* locus and identify other mating-type and pathogenicity related genes.

Results

DNA from three different loci hybridize to 23 unique BAC clones

In consecutive hybridizations using sequences from the *a1* and *b2* gene clusters of *U. hordei* and the *U. maydis hgl1* locus, several clones were identified using the same high density nylon filter (Figure AI.1; Table 2.2; Genome Systems, Inc). The filter was stripped of each probe between hybridizations (by submersion in a boiling solution containing 1% SDS) to avoid

A



B

BAC clones identified		
aw-1	**bw**	**hgl**
N17-6	C14-2	C8-1
N12-1	D9-5	E15-2
N9-5	F7-4	L18-6
I2-6	H7-3	L20-5
F11-3	H8-6	M22-5
E10-4	I3-4	O14-4
B24-2	K9-6	P5-5
	N10-6	P18-6

Figure AI.1. *U. hordei* BAC clones identified by DNA hybridization. DNA blots hybridized with *U. maydis* probes aw-1, bw and hgl (A; see Table 2.2). The clones identified in A were labeled according to their well position (B).

confusion in clone identity and origin. These experiments revealed a seven- to eight-fold redundancy in the BAC library because seven clones were identified using probe **aw-1** and eight clones using probes **bw** and **hgl**. The BAC clones were named according to their position in the six 96-well plates supplied; the first letter and number combination refers to the well position and the number following the dash denotes the plate number (Figure AI.1). For example, probe **aw-1** hybridized to clone N17-6, indicating that this clone is located on plate number six, in the well at the intersection of row N and column 17.

Discussion

Using pulse-field gel electrophoresis and hybridization analysis, a 500-kb region separating the *a* and *b* gene complexes of *U. hordei* was discovered (see Chapter 3). These experiments revealed the position of the *MAT* locus on the chromosome and the minimum size of the locus, however, the possibility remains that the mating-type locus may extend beyond the markers defining the *a* and *b* gene clusters. To investigate the boundaries of the *MAT* locus, BAC clones containing the *a* and *b* gene complexes were isolated with the idea that sequences from these clones could serve as markers to map the extent of recombination suppression beyond the gene complexes.

Hybridizations using probes **aw-1** and **bw** failed to show cross-hybridization between BAC clones, supporting the previous findings of a large intervening region between *a* and *b* (Figure AI.1). The BAC clones were characterized by restriction enzyme digestion and used to generate BAC end probes. Selected BAC end probes were then used to identify RFLP's between parental strains 4857-4 and 4857-5. Despite the use of 14 different restriction enzymes to digest the parental genomic DNA, the probes generated failed to reveal any polymorphisms between parental strains. The identification of BAC clones carrying the *a* and *b* gene complexes have

aided in the construction of a BAC fingerprint-based physical map of the chromosome carrying *MAT* (Figure AI.2). Interestingly, current efforts to identify RFLP markers have provided evidence for the presence of large amounts of repetitive DNA on the *MAT* chromosome (G. Jiang, personal communication).

The suppression of recombination among a cluster of genes involved in mating and pathogenicity may indicate that other genes involved in these process are present at this locus. To explore this possibility, sequences from the *hgl1* and *prf1* loci of *U. maydis* were used to identify BAC clones containing homologous sequences from *U. hordei*. Probe **hgl** hybridized to eight unique clones, suggesting that the *U. hordei hgl1* homolog is not closely associated with either the *a* or the *b* gene complexes. In fact, the localization of the *hgl1* and *MAT* containing BAC clones to two different contigs was confirmed by BAC fingerprint mapping (G. Jiang, personal communication). However, the BAC fingerprint map has not been completely assembled and the contig containing the *MAT* locus does not represent the entire chromosome. It is possible that the contig carrying the *U. hordei hgl1* homolog represents the "right" arm of the mating-type chromosome beside the *b* gene complex (Figure AI.2). Using blots of CHEF gels electrophoresed for 48 hours, the *U. maydis hgl1* gene was shown to hybridize to a high molecular weight chromosome that either represents or co-migrates with the *MAT* chromosome (Figure AI.2). Hybridization experiments with the *U. maydis* **prf1** probe were unsuccessful because only a high level of background hybridization could be detected. However, sequence analysis of the **prf1** probe showed that this probe did not actually originate from the *U. maydis prf1* locus and the possibility that a *U. hordei prf1* homolog is present within the *MAT* locus remains. In fact, recent sequencing efforts have shown that the *MAT* locus contains multiple genes with high homology to genes regulating mating and pathogenicity in other fungal species (G. Jiang, personal communication).

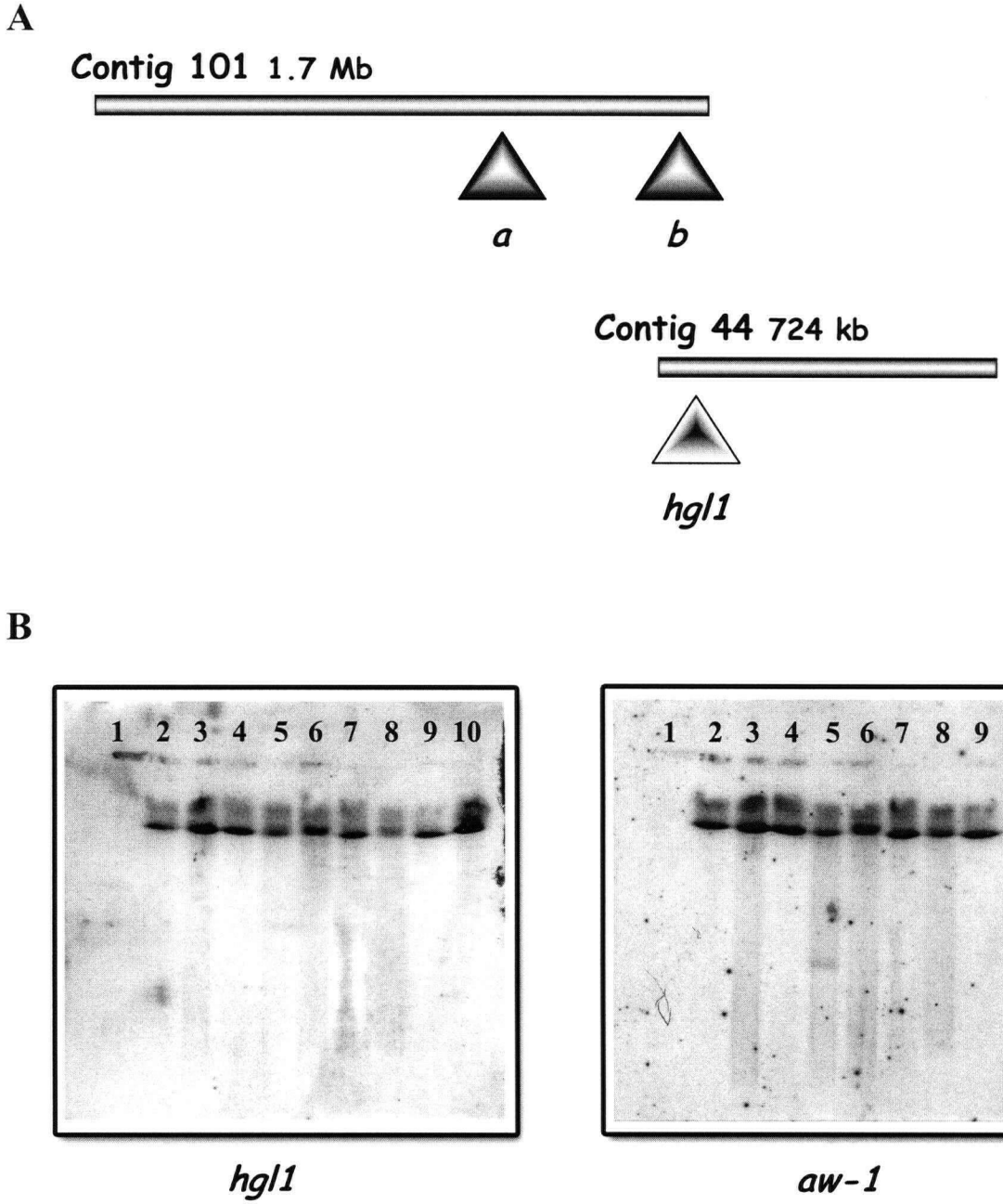


Figure AI.2. The BAC clones identified by hybridization with probes from the the *a*, *b* and *hgl1* loci are located on two contigs (A). DNA gel blots of a CHEF gel hybridized with the **hgl1** probe (hybridization with probe **aw-1** is shown for comparison) (B). Lanes: 1, *S. cerevisiae* chromosome marker; 2, I-*Sce*I digested 4857-4 (*a1b1*); 3, I-*Sce*I digested 364-86 (*a1b1*; single tag at *a1*); 4, undigested 364-86dt21 (*a1b1*; double tag at *a1*, *b1*); 5, I-*Sce*I digested 364-86dt21 (*a1b1*; double tag at *a1*, *b1*); 6, I-*Sce*I digested 365-57dt51 (*a2b2*; double tag at *a2*, *b2*); 7, undigested 365-57dt51 (*a2b2*; double tag at *a2*, *b2*); 8, I-*Sce*I digested 365-57 (*a2b2*; single tag at *b2*); 9, I-*Sce*I digested 4857-5 (*a2b2*); 10, *U. maydis* strain 521. See Figure 3.2 for **aw-1** probe location and Table 2.2 for details on probe **hgl**.

APPENDIX II: List of Suppliers

Supplier	Location	Fax number
Amersham Pharmacia Biotech	Piscataway, New Jersey	877-295-8102
Bio-Rad Laboratories	Hercules, CA	800-879-2289
Böehringer Mannheim		
Calbiochem	San Diego, California	800-776-0999
Carl Zeiss Microimaging	Thornwood, New York	914-681-7446
Invitrogen	Burlington, Ontario	800-387-1007
Cayla	Toulouse, France	+33 (0)5 62 71 69 30
Difco (Fisher Scientific)	Nepean, Ontario	1-800-463-2996
New England Biolabs Inc.	Beverly , MA	978-921-1350
Perkin-Elmer	Shelton, Connecticut	203-925-4654
Promega Corp.	Madison , WI	608-277-2601
Roche Molecular Biochemicals	Indianapolis , IN	800-428-2883
Sigma	Mississauga, Ontario	800-325-5052
Werner BioAgents	Jena-Cospeda, Germany	+49 3641 423729