ALIGNMENT BETWEEN GENETIC AND PHYSICAL MAP, AND PHEROMONE FUNCTIONS IN *GIBBERELLA ZEAE*

by

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M. S., Seoul National University, 2001

AN ABSTRACT OF A DISSERTATION

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Abstract

Gibberella zeae is an ascomycete filamentous fungus and the major cause of Fusarium head blight, also called scab, in small grains. This dissertation contains three related studies of *G. zeae*. In the first, the genetic map was aligned with the first assembly of the genomic sequence released by The Broad Institute (Cambridge, MA). Approximately 99% of the sequence was anchored to the genetic map, indicating the high quality of the sequence assembly and validity of the genetic map. The alignments grouped the linkage groups and supercontigs into four sets, which is consistent with the hypothesis that there are four chromosomes in this fungus. In the second, the sex pheromone precursor genes (*ppg1* and *ppg2*) and the pheromone receptor genes (*pre1* and *pre2*) were identified and characterized. Deletion of *ppg1* or *pre2* (*Δppg1* or *Δpre2* strains) reduced the number of perithecia produced by self-fertilization, but did not completely block perithecial formation. The proportion of crosses resulting from outcrossing increased when the *Δppg1* strains were used as the female in crosses with male strains containing an intact *ppg1* gene. *Δppg2* and *Δpre1* mutants had no discernable effect on morphological phenotype or self-fertilization. Thus, one of the pheromone/receptor pairs (*ppg1*/*pre2*) found in many Ascomycetes has a role in, but is not essential for, selfing or outcrossing in *G. zeae*, whereas the other pheromone/receptor pair (*ppg2*/*pre1*) no longer has a detectable function in sexual reproduction. In the third study, spore germination of *G. zeae* was tested in the presence of α-factor-like pheromone peptides of *G. zeae* or *N. crassa*. The pheromone peptide of *N. crassa* more efficiently inhibited spore germination than did the peptide from *G. zeae*. Arginine and lysine residues were the most important determinants in blocking spore germination. In conclusion, this research has validated the genetic map and the genomic assembly of *G. zeae*, characterized sex pheromone functions and characterized pheromone peptide ability to inhibit spore germination. The pheromone peptides of *G. zeae* and *N. crassa* may be useful as control agents for *G. zeae* and pheromone peptide efficacy might be further enhanced by judicious substitutions for some of the amino acids.

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CHAPTER 1 - LITERATURE REVIEW

Gibberella zeae

Plant Pathogen: The ascomycete fungus *Gibberella zeae* (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) causes Fusarium head blight (FHB), also called scab, in wheat, barley, and other grains, stalk and ear rot of corn, a root rot of cereals, and stem rot with stub dieback in ornamental plants like carnation. FHB was first described over a century ago and was considered an important factor limiting production of wheat, barley, and other small grains. It has the ability to completely destroy a crop within a few weeks (Dickson and Mains, 1942; McMullen *et al.,* 1997; Stack, 1999). In North America, *G. zeae* predominates among *Fusarium* species that cause FHB, while other species may predominate in cooler climates or where crops other than wheat and corn are dominant (Nelson *et al.,* 1983; Sutton, 1982; Tschanz *et al.,* 1976; Wong *et al.,* 1992). In the United States, FHB has occurred at epidemic levels in several years during the last decade, and direct and secondary losses were estimated to be $2.7 billion from 1998 to 2000 (Nganje *et al.,* 2002).

Disease cycle: *G. zeae* overwinters and survives in infected grain, grass stubble, and cornstalk residue as mycelia, conidia, and perithecial initials (Sutton, 1982). Also this fungus can contaminate seeds internally and externally (Markell and Francl, 2003). The conidia are produced abundantly during warm and moist conditions on corn and small grain residue and ascospores are discharged into air under humid conditions in the spring (Sutton, 1982; Trail *et al.,* 2002). The infection is initiated by ascospores or conidia in wheat florets during the short period (10-20 days) from anthesis through the soft dough stage of kernel development (Schroeder and

Christensen, 1963). Early researchers reported that anthers were the initial infection site (Adams, 1921), but recent studies found that hyphae may directly penetrate ovaries or the inner walls of lemmas, paleas, or glumes (Bushnell *et al.,* 2003). Infections are most serious when the anthers are exposed during flowering (Sutton, 1982). Symptoms develop within three days after infection when the temperature is between 25 and 30°C with high humidity. Symptoms of FHB begin at the point of infection with water-soaked brown spots. Bleaching of the original spikelet becomes apparent within a few days (Parry *et al.,* 1995). Subsequently, the fungus may colonize adjacent spikelets via the rachis and kill all or part of the spike. In seven to ten days after infection, salmon-pink conidia form on the bases of the infected spikelets and these conidia may cause secondary infections. Perithecia containing ascospores are sometimes produced on the heads late in the season (Lipps, 1996; Malvick, 1996).

Genetics: *G. zeae* is homothallic (i.e., self-fertile; Eide, 1935), but it can be outcrossed under laboratory conditions (Bowden and Leslie, 1999). Despite homothallism, genetic heterogeneity in field populations of this fungus suggests that outcrossing occurs frequently in the field (Bowden and Leslie, 1992; Walker *et al.,* 2001). Sexual reproduction in ascomycete fungi is controlled by a mating type locus (*MAT*) (Glass *et al.*, 1988; Nelson, 1996; Coppin *et al.*, 1997; Turgeon, 1998; Figure 1.1). In most filamentous fungi, there are two structurally unrelated alleles (idiomorphs) at the *MAT* locus. The structure of the *G. zeae MAT* locus is similar to that of other heterothallic filamentous fungi but both *MAT* idiomorphs are tightly linked on the same chromosome and are not allelic (Yun *et al.,* 2000; Figure 1.1A). Functional versions of both mating type genes in *G. zeae* is required for self-fertility and the deletion of either *MAT1-1* or *MAT1-2* results in the loss of homothallism and renders the fungus effectively heterothallic (Lee *et al.,* 2003).

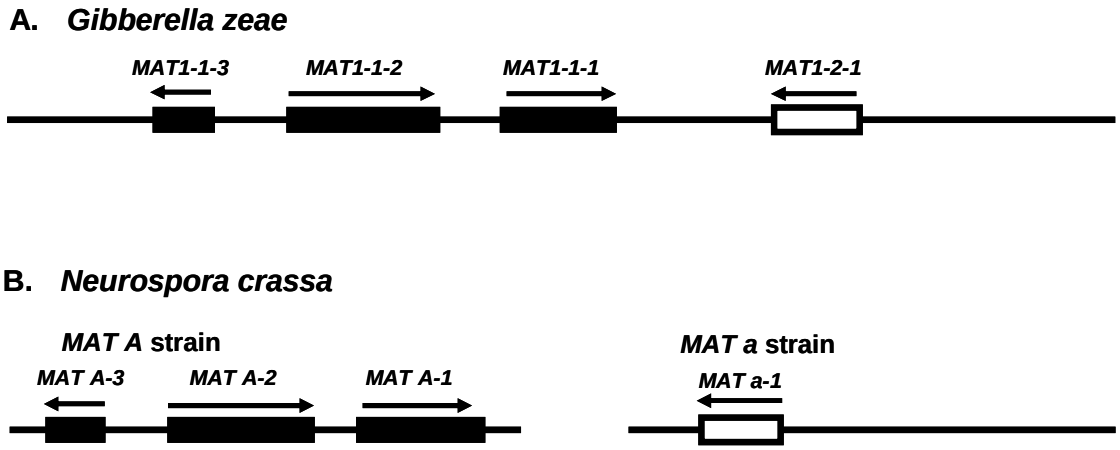

Figure 1.1 Organization of *MAT* loci. (A) *MAT1-1* and *MAT1-2* loci were tightly linked on the same chromosome in the homothallic fungus *Gibberella zeae* (Yun *et al*., 2000). (B) In the heterothallic fungus *Neurospora crassa*, mating type of each fungal strain is determined by *MAT A* or *MAT a* alleles (Coppin *et al*., 1997).

A genetic map of *G. zeae* was constructed by crossing *G. zeae* strain Z-3639 and R-5470 isolated from Kansas and Japan, respectively (Jurgenson *et al.,* 2002). The genetic map had nine linkage groups with 1048 polymorphic markers mapped to 468 unique loci. The total map length is about 1300 cM and is based on AFLP markers. Gale *et al.* (2005) constructed a second genetic map from a cross between PH-1 (NRRL 31084) isolated from Michigan and a closely related strain from Minnesota (NRRL 34097). This genetic map has 235 loci with nine linkage groups and 1234 cM, and is based on CAPs, VNTR, and AFLP markers.

Mycotoxins: In addition to direct yield loss by *G. zeae*, harvested grain may be significantly contaminated by trichothecene mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV), and the estrogenic mycotoxin, zearalenone, which are hazardous to animals (McMullen *et al.,* 1997). Trichothecenes are sesquiterpenoids that are potent inhibitors of eukaryotic protein biosynthesis, and cause food refusal, diarrhea, emesis, alimentary hemorrhaging and contact dermatitis in animals, and are linked to alimentary toxic aleukia and Akakabi toxicosis, illnesses characterized by nausea, vomiting, anorexia and convulsions in humans (Desjardins, 2006).

G. zeae strains may be divided into two chemotaxonomic groups based on production of 8-ketotrichothecenes: type I able to form DON with its acetylated derivatives and type II which produces NIV (Anderson *et al.,* 1989; Ichinoe *et al.,* 1983; Yoshizawa *et al.,* 1977). There are regional differences in the incidences of the two chemotypes in strains isolated from cereals. The levels of NIV detected in barley and wheat in Korea and Japan were several times higher than those of DON, whereas DON is the major contaminant in Argentina, North America, China, Poland, and Germany (Kim *et al.,* 1993; Sutton, 1982).

Biosynthesis of trichothecenes has been characterized in the fungus *Fusarium sporotrichioides* and the pattern is similar in *G. zeae* except for the acetylation (Lee *et al.,* 2002) and oxygenation patterns (Lee *et al.,* 2002; McCormick *et al.,* 2004; Meek *et al.,* 2003). Many of the trichothecene biosynthesis genes are tightly linked in a gene cluster. The cluster includes trichodiene synthetase (*Tri5*) (Hohn *et al.,* 1989), P450 oxygenase (*Tri4* and *Tri11*) (Alexander *et al.,* 1988; Hohn *et al.,* 1995), acetyltransferase (*Tri3* and *Tri7*) (Brown *et al.,* 2001; Lee *et al.,* 2002; McCormick *et al.,* 1996), transcription factors (*Tri6* and *Tri10*) (Proctor *et al.,* 1995; Tag *et al.,* 2001), a toxin efflux pump (*Tri12*) (Alexander *et al.,* 1999), and hypothetical uncharacterized proteins (*Tri8, Tri9,* and *Tri14*) (Hohn *et al.,* 1998; McCormick *et al.,* 1998; Dyer *et al.,* 2005). Another acetyltransferase gene (*Tri101*) (Kimura *et al.,* 1998) is unlinked to the cluster.

Zearalenone (ZEA) is a polyketide metabolite that causes estrogenic disorders in swine ingesting *Fusarium*-contaminated maize, wheat, barley and sorghum (Mirocha and Christensen, 1974). Molecular genetics of the biosynthesis of ZEA in *G. zeae* has not been described extensively and two different polyketide synthase genes are involved in its biosynthesis (Kim *et al.,* 2005; Lysøe *et al.,* 2006).

Pathogenicity factors: Disruptants of the *Tri5* gene (Proctor *et al.,* 1995, 1997), which encodes the first enzyme in the trichothecene biosynthesis pathway (Hohn *et al.,* 1989), or *Mgv1* gene, which encodes a mitogen-activated protein kinase and is homologous to the *Mps1* gene of the rice blast fungus *M. grisea* (Hou *et al.,* 2002), have reduced virulence. These results suggest that trichothecene production and *Mgv1* contribute to the virulence of *G. zeae* on wheat but are not essential for pathogenicity. The only pathogenicity factor known so far is the *Map1* gene, which is a homolog of a pathogenicity gene of *M. grisea,* the mitogen-activated protein kinase

gene *Pmk1* (Nishida and Gotoh, 1993; Xu and Hamer, 1996). *Map1* disruption mutants in *G. zeae* lost their ability to form perithecia and pathogenicity (Urban *et al.*, 2003).

Species concepts: Two populations of *F. graminearum* were recognized based on distinct ecological traits (Burgess *et al.*, 1975; Francis and Burgess, 1977). Group I isolates did not form perithecia in culture and were thought to be heterothallic. They are primarily soilborne and have been found in arid regions of Australia, South Africa, and North America. The Group I strains were described as *Fusarium pseudograminearum* (Aoki and O'Donnell, 1999). Group II is homothallic and produces abundant perithecia in the field and under laboratory conditions (Bowden and Leslie, 1992, 1999). Group II was divided into seven phylogenetic lineages based on DNA sequences of six structural genes (O'Donnell *et al.*, 2000) and recently was subdivided into nine phylogenetic species based or the genealogical concordance phylogenetic species recognition (GCPSR) concept (O'Donnell *et al.*, 2004).

Fertility barriers between the lineages (or species) and lineage 7 have not been found (Bowden *et al.*, 2004; Bowden *et al*., 2006) and putative interlineage hybrids have been found in field populations in Korea, Uruguay and southern Brazil (Leslie and Bowden, 2005). Both laboratory crosses and the existence of putative hybrids under field conditions suggest that significant gene flow could occur between the lineages if different lineages are present at the same location (Leslie and Bowden, 2005). Pending resolution of these biological questions, Leslie and Summerell (2006) recommended using the single name *Fusarium graminearum* for all members of the *F. graminearum* clade proposed by O'Donnell *et a*l. (2004).

Expressed sequence tags and genomic sequence: Analysis of expressed sequence tags (ESTs) was performed in *G. zeae* (Trail *et al.*, 2003). The EST database contains 2,110 predicted genes from carbon-/nitrogen-starved mycelia and cultures of maturing perithecia.

Whole genome shotgun sequencing was performed by the Broad Institute, Center for Genome Research (www.broad.mit.edu/annotation/fungi/*Fusarium*/). The sequence has approximately 10 × coverage of the genome and the total size of the assembly is about 36 Mb. The *F. graminearum* genome contains ~ 11,600 predicted genes and 511 contigs in 43 scaffolds.

Initial Interaction for Fertilization in Fungi

The initial interaction between male and female structures during sexual fertilization has been well described in *N. crassa*. Although filamentous ascomycete fungi do not have differentiated sexes, most of them have clearly defined mating types and strains that are self-sterile hermaphrodities (Backus, 1939; Cayley, 1930; Dodge, 1932; Shear and Dodge, 1927). In *N. crassa*, both mating types constitutively produce protoperithecia, the female structures, that develop into perithecia following fertilization with a nucleus of the opposite mating type. Special hyphae called trichogynes from the protoperithecia are used to retrieve the male nuclei for fertilization. Asexual spores or hyphal fragments may serve as the source of the male nucleus. Bistis (1981) found that there are chemotropic interactions between trichogynes and conidia of opposite mating-type in *N. crassa*. When a conidium from an opposite mating type is placed near a protoperithecium, the trichogyne changes its growth to reach the conidium with the nucleus of the conidium eventually moving into the protoperithecium through the trichogyne. Once fertilized, the protoperithecium developed into a mature perithecium that contains thousands of sexual spores, termed ascospores. The chemotropic interaction suggests that the conidia constitutively secrete a pheromone-like substance and that protoperithecia have a mechanism to recognize the substance.

Pheromone Response Pathway in Yeast

The recognition between male and female structures is triggered by an interaction between sex pheromone peptides and pheromone receptors in the ascomycete *Saccharomyces cerevisiae* (yeast hereafter). Yeast can exist as two haploid cell types, a and α, which are morphologically indistinguishable but which can be distinguished by their ability to produce and respond to extracellular signaling molecules (Herskowitz, 1986). The a mating type cells secrete a-factor and encode a receptor for α-factor, whereas α mating type cells secrete α-factor and encode a receptor for a-factor. Interactions between these pheromones and receptors on cells of the opposite mating type cause a set of responses, including cell cycle arrest in the G1 phase, morphological alteration, induction of cell surface agglutinins, and stimulation of transcription of certain genes (Manney *et al.,* 1981; Sprague *et al.,* 1983).

α-factor: α-factor is encoded by two similar structural genes, *MFα1* and *MFα2* (Kurjan and Herskowitz, 1982; Singh *et al.,* 1983). The α-factor precursors contain tandem repeats of α-factor sequence (Kurjan and Herskowitz, 1982). The precursors are glycosylated and processed within a classical secretory pathway to produce mature α-factor: Yeast *Kex2* protease recognizes lys-arg doublets and is involved in C-terminal processing of pheromone repeats. C-terminal lys-arg residues exposed by *Kex2* protease are removed by *Kex1* carboxypeptidase. The mature pheromone is an extracellular oligopeptide, 13 residues long (Fuller *et al.*, 1986; Julius *et al.,* 1983).

a-factor: The structure of a-factor is characterized by its hydrophobic nature that results from an isoprenyl group on the C-terminal cysteine residue. The *MFa1* and *MFa2* genes encode small precursors for two forms of a-factor, both of which are 12 residue lipopeptides (Anderegg

et al., 1988; Betz *et al.,* 1987). The precursors undergo complex post-translational processing: S-farnesylation at the cysteine residue by Ram2p and Ram1p (farnesyltransferase α and β, respectively), proteolysis of AAX residues by Rce1p, carboxymethylation of the exposed cysteine by Ste14p (methyltransferase), and two step proteolysis of the amino terminus by Ste24p (aminopeptidase 1) and Ste23p (aminopeptidase 2). The mature pheromone is secreted directly across the membrane via Ste6p, an ATP-dependent transporter (Davey *et al.,* 1998; Kurjan, 1993; Michaelis *et al.,* 1992).

Pheromone receptors: Ste2p and Ste3p are the α-factor and a-factor receptors, respectively (Jenness *et al.,* 1983; Hagen *et al.,* 1986). *Ste2* provides the specificity for the response to the different α-factors produced by *S. cerevisiae* and *S. kluyveri* (Marsh and Herskowitz, 1988). Expression of *Ste2* in α cells allows response to α-factor and expression of *Ste3* in a cells allows response to a-factor (Bender and Sprague, 1986). *Ste2* and *Ste3* contain seven transmembrane (TM) domains, an extracellular N-terminus and a cytoplasmic C-terminus (Cartwright and Tipper, 1991; Hagen *et al.,* 1986). These receptors are coupled to a heterotrimeric G-protein complex consisting of Gα (Gpa1p) bound to GDP and Gβγ (Ste4p and Ste18p). The binding of pheromone to its complementary receptor causes exchange of GDP for GTP on Gα, which releases Gβγ to propagate the signal and activate a mitogen-activated protein kinase (MAPK) signal transduction pathway (Ste11p, Ste7p, Fus3p). The MAPK pathway ultimately activates the transcription factor Ste12p, which promotes the expression of over 200 genes to mediate various mating specific outputs (Robert *et al.,* 2000).

Functions of Pheromones and Receptors in Filamentous Fungi

In the heterothallic ascomycete *N. crassa*, *mfa-1*, an a-factor-like pheromone precursor gene, has multiple functions (Kim *et al.,* 2002) including the attraction of trichogynes, female

sexual development and ascospore production by strains of both mating types, and vegetative growth in strains of both mating types. Deletion of the *pre1* gene, a receptor for the *mfa-1* peptide, results in female sterility in crosses with *MAT-A* because the chemotropic growth of trichogynes towards the *MAT-a* conidia no longer occurs (Kim *et al.,* 2004). In *C. parasitica*, *mf1-1*, the α-factor-like pheromone precursor gene, is required for male fertility but does not affect either vegetative growth or female fertility (Turina *et al.,* 2003).

In the homothallic ascomycete *A. nidulans*, pheromone receptor genes (*gprA* and *gprB*) are required for self-fertilization. Deletion of each gene reduces the number of ascospores produced, and deletion of both genes blocks homothallic development (Seo *et al.*, 2004). Two pheromone precursor and receptor genes are expressed in the homothallic ascomycete *S. macrospora* (Pöggeler, 2000). Deletion of any individual gene did not trigger phenotypic change, but double deletions reduced self-fertility (Mayrhofer *et al.,* 2006).

In some fungi, pheromones play a role in post-fusion events as well as in cell-cell recognition and fusion; the induction of meiosis in *Schizosaccharomyces pombe* (Chikashige *et al.,* 1997), stimulation of filamentous growth in *Ustilago maydis* (Debuchy, 1999), and internuclear recognition in *Schizophyllum commune* and *Podospora anserina* (Spellig *et al.,* 1994).

Pheromone-Related Inhibition of Conidial Germination and Mating

In yeast, pheromone induces the competence of responsive cells to mate with cells of the opposite mating type by causing G1 cell cycle arrest and differentiate into mating-competent gametes (Herskowitz, 1995). Yeast α-factor inhibits appressorium formation by *M. grisea* in a mating type-specific manner and protects plants from *Mat1-2* strains (Beckerman *et al.,* 1997).

In *N. crassa*, macroconidial germination was inhibited when the macroconidia were placed near protoperithecia formed by a strain of the opposite mating type (Bistis, 1981).

Mating inhibition by pheromone-related compounds has been described in *S. pombe* (Davey, 1991), *U. maydis* (Spellig *et al.*, 1994), and *U. hordei* (Kosted *et al.*, 2002; Sherwood *et al.*, 1998). The *S. pombe* and *U. hordei* compounds were oxidation products of the pheromones (Davey, 1991; Kosted *et al.*, 2002). Similar compounds were found in *S. cerevisiae* extracts (Betz *et al.*, 1987) and these compounds had less activity than the native pheromone peptide (Caldwell *et al.*, 1994; Marcus *et al.*, 1991).

Although the mechanisms by which mating pheromone peptides inhibit conidial germination are not known, competition for receptor binding (Beckerman *et al.*, 1997; Zhang *et al.*, 1994), loss of a pheromone gradient (Dorer *et al.*, 1995), and transport inhibition of the pheromone (Zhang *et al.*, 1994) have all been suggested as possible explanations..

Objectives

The objectives of this study were (1) to align the genetic map with the physical assembly of *G. zeae*, (2) to characterize the functions of pheromone precursors and pheromone receptors in *G. zeae*, and (3) to check spore germination inhibition by pheromone peptides as a possible new control method against Fusarium head blight.

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CHAPTER 2 - ALIGMENT BETWEEN GENETIC AND PHYSICAL MAP IN *GIBBERELLA ZEAE*

Abstract

Jurgenson *et al.* (2002) previously published a genetic map of *Gibberella zeae* (*Fusarium graminearum*) based on a cross between Kansas strain Z-3639 (lineage 7) and Japanese strain R-5470 (lineage 6). The genetic map was based on 1048 AFLP markers and consisted of nine linkage groups. We aligned the genetic map with the first assembly of the genomic sequence of *G. zeae* strain PH-1 (lineage 7) that was released by The Broad Institute (Cambridge, MA). We used 7 sequenced structural genes and 108 sequenced AFLP markers from all nine linkage groups (LG) of the genetic map and aligned them with nine supercontigs (SC) of the genomic sequence. LG1, LG7, LG8 and LG9 aligned with SC2 and SC5; LG2 aligned with SC3, SC8 and SC9; LG 3 aligned with SC4 and SC6; and LG4, LG5 and LG6 aligned with SC1 and SC7. The nine linkage groups in the previous genetic map were reduced to six linkage groups, and the total size of genetic map decreased from 1286 to 1140cM. Eight markers had no-hits on the genome assembly and were located in regions that correspond to the ends of supercontigs. Four of these markers were linked and mapped to the end of LG2, which suggests that at least 155 kb of genomic sequence could be missing from the end of SC3 in the genome assembly. Approximately 99% of the sequence was anchored to the genetic map, indicating the high quality of the sequence assembly and the relative completeness and validity of the genetic map. The alignments grouped the linkage groups and supercontigs into four independent sets, which is consistent with the hypothesis that there are four chromosomes in this fungus. The alignment of

genetic and physical maps was generally excellent, but two nonsyntenous regions were consistent with putative inversion regions on LG2 and LG4 as suggested by Jurgenson *et al.* (2002).

Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is the most important causal agent of Fusarium head blight (FHB) of cereals (Leonard and Bushnell, 2003). FHB has recently re-emerged worldwide as a devastating disease of wheat and barley (McMullen *et al.*, 1997). In addition to direct yield losses, FHB reduces grain quality (Seitz *et al.*, 1986), and infected seed is often contaminated with mycotoxins such as nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) (Marasas *et al.*, 1984; Mirocha and Christensen, 1974). In the United States, direct and secondary losses were estimated to be $2.7 billion from 1998 to 2000 (Nganje *et al.*, 2002).

G. zeae is a homothallic (i.e. selfing) Ascomycete fungus (Eide, 1935) but it can also outcross. For genetic studies, special methods are required to distinguish selfed from outcrossed progeny (Bowden *et al.*, 2004). Bowden and Leslie (1999) developed a laboratory outcrossing method using nitrate non-utilizing (*nit*) mutants of different *G. zeae* strains, which eventually led to several genetic maps and QTL studies of *G. zeae* (Jurgenson *et al.*, 2002; Gale *et al.*, 2005; Cumagun *et al.*, 2004). Lee *et al.* (2003) overcame the problem of homothallism in crosses by partially disabling the mating type (*MAT*) genes. In addition to outcrossing under laboratory conditions, high levels of genotypic heterogeneity in field populations of this fungus suggest that outcrossing frequently occurs in nature (Bowden and Leslie, 1992; Zeller *et al.*, 2004).

The genome of *F. graminearum* was sequenced at the Broad Institute (Cambridge, MA) with *F. graminearum* strain PH-1 (lineage 7) and was publicly released in 2003

(http://www.broad.mit.edu/annotation/fungi/Fusarium/). The first genome assembly contains 36 Mb total length, 11,640 annotated genes, 511 contigs > 2 kb, and 43 supercontigs, with more than 10 × genomic coverage.

The first genetic map of *G. zeae* (Jurgenson *et al.,* 2002) was constructed from an outcross between *nit* mutants of Z-3639 (lineage 7) and R-5470 (lineage 6; termed *F. asiaticum* by O'Donnell *et al.,* 2004). The map was saturated with 1048 polymorphic markers that mapped to 468 unique loci on nine linkage groups. The total map length was 1286 cM with an average interval of 2.8 map units between loci. Toxin type produced (DON or NIV) was controlled by a single locus linked to trichodiene synthetase (*TRI5*). About half of the genes required for trichothecene biosynthesis are located in a 25-kb trichothecene gene cluster (Kimura *et al.,* 2001). The genetic conclusions were confirmed by Lee *et al*. (2002), who showed that the *TRI7* and *TRI13* genes determine which toxin is produced and that these loci are located in the trichothecene cluster near *TRI5*.

The genetic map also was used to map loci for pathogenicity and aggressiveness of *G. zeae* towards wheat (Cumagun *et al.,* 2004). A single QTL for aggressiveness of *G. zeae* was detected on linkage group 1 and was linked to the *TRI5* locus, consistent with previous reports that trichothecene-deficient *G. zeae* mutants were less aggressive than wild type strains (Proctor *et al.,* 1995). Pathogenicity (*PATH1*) segregated qualitatively and was tightly linked to *PER1* (a locus for perithecial production), *PIG1* (a locus for red pigmentation), and *TOX1* (a locus for the amount of toxin produced).

Several questions remain regarding this genetic map: (1) Why do five of the nine linkage groups show segregation distortion? (2) Why is the distribution of crossovers across the linkage groups apparently not random? (3) Are unusual regions in two of the linkage groups large

heterozygous inversions? (4) Are *PATH1, PER1, PIG1,* and *TOX1* the same locus? (5) Where are *nit-1* and *nit-3* (the heterozygous loci used to force the cross)?

A second map of *G. zeae* (Gale *et al.,* 2005) was based on cross between two closely related *F. graminearum* strains PH-1 and 00-676. This map had 235 loci with nine linkage groups and a total length of 1234 cM. The second map was aligned with the first assembly of the genomic sequence of *G. zeae.* The nine linkage groups aligned with 22 supercontigs and were combined into four groups that were postulated to represent chromosomes (Gale *et al.,* 2005).

Our objectives in this study were (1) to validate the first genetic map of *G. zeae* (Jurgenson *et al.*, 2002) and resolve questions arising from that map, (2) to validate the proposed sequence assembly, (3) to estimate chromosome number and size, and (4) to compare the first Jurgenson *et al.* (2002) map with that of Gale *et al.* (2005).

Materials and Methods

Genetic map and genomic sequence assembly: The genetic map of *G. zeae* based on the cross of strains Z-3639 and R-5470 (Jurgenson *et al*., 2002) and the physical genomic assembly of *G. zeae* strain PH-1 of the Broad Institute (Cambridge, MA; http//www.broad.mit.edu/annotation/fungi/Fusarium/) were used in this study.

Isolation and reamplification of AFLP fragments: The protocol of Shan *et al.* (1999) was modified slightly to amplify AFLP markers already placed on the genetic map. For each primer pair, AFLP gels were run using genomic DNA of *G. zeae* strains Z-3639, R-5470, and PH-1, and polymorphic bands were correlated with markers from the map. Dried AFLP gels and x–ray films were aligned and needles used to mark the polymorphic fragments. A razor blade was used to excise the portion of the gel containing the targeted DNA. Each gel slice was placed in a PCR tube with 20 µl of sterile water overnight at 4ºC. Two µl of each sample was used for

reamplification in a MJ PTC-200 Thermocycler (MJ Research, Watertown, MA). The reaction was performed in a total volume of 30 µl as; 1 min at 94ºC, followed by 30 cycles (30 sec at 94ºC, 1 min at 56ºC, and 1 min at 72ºC) with a final extension at 72ºC for 10 min. The corresponding unlabeled selective AFLP primers were used to prime the reaction. A 10 µl aliquot of each PCR product was resolved on a 1.5% agarose gel to estimate band size and number.

Sequencing of AFLP fragments and selection of clones: Thirty-one reamplified PCR products were sequenced directly with an ABI3700 DNA Analyzer in the DNA sequencing facility at Kansas State University, after purification with a PCR clean-up system (Promega, Madison, WI). Ninety-eight fragments were cloned into a pGEMT-easy vector (Promega) by using a kit and following the manufacturer's instructions. Three clones were selected and sequenced for each of these 98 fragments.

For selected markers, we designed extended AFLP primers with three additional bases on each end to confirm polymorphism in the progeny. These PCR reactions were performed under the same conditions as described above with annealing temperatures of 59ºC to 64ºC depending on primer pair.

The program AFLPs in silico (Rombauts *et al.,* 2003) was used with the *G. zeae* PH-1 genomic database to generate virtual AFLP marker bands. Sequenced clones were compared with bands from the virtual AFLP analysis to confirm their presence and size.

Alignment of two maps: The sequenced genetic markers were found in the first genome assembly of *G. zeae* with blastn (Altschul *et al.,* 1990), and the genetic map and the genome assembly were aligned manually.

Mapping of nuclear genes: Seven structural genes, elongation factor (*EF-1α*), phosphate permease (*PHO*), trichodiene synthetase (*TRI5*), trichothecene 3-O-acetyltransferase (*TRI101*), a regulator for trichothecene biosynthesis (*TRI15*), a putative reductase (*RED*), and mitogen-activated protein kinase (*MGV1*), were mapped. *TRI5* was mapped previously by using restriction fragment length polymorphisms (RFLPs) (Jurgenson *et al.,* 2002).

Six genes were mapped by cleaved amplified polymorphic sequences (CAPS). Each gene was amplified from both parental strains R-5470 and Z-3639, with specific primers. Except for *MGV1* primer sets, all of the primers used were those of O'Donnell *et al.* (2000). Primers for *MGV1* were designed this study (forward primer: 5′-CACAAATACACCATGGGCGACCTA-3′, reverse primer: 5′-GACACCCTGGCCCTGAAGACCTC-3′) and synthesized by IDT (Coralville, IA). Amplified DNA fragments were sequenced after purification with a PCR clean-up system (Promega, Madison, WI). Sequences from both parental strains were compared to identify polymorphic restriction sites. CAPS markers for each gene were checked in all 99 progeny of the mapping population used to construct the first genetic map (Jurgenson *et al.,* 2002). The genes also were placed on the genetic map by using Map Manager QTX11 (http://mapmgr.Roswellpark.org/mm PTX) on a Macintosh G4 Power PC computer (Manly and Olson, 1999).

Location of *nit1* and *nit3*: To localize *nit1* and *nit3* of *G. zeae* in the genomic database, *nit-3* (nitrate reductase; GI 548358) and *nit-4* (nitrogen assimilation transcription factor; GI 128353), the homologs of *nit1* and *nit3* in *Fusarium* (Klittich and Leslie, 1988), respectively, from *Neurospora crassa* were blasted on the Fusarium genome database.

Switching of upstream regions of *MGV1* genes between R-5470 and Z-3639: Loci for pathogenicity (*PATH1*), perithecial production (*PER1*), red pigmentation (*PIG1*), and amount of

toxin produced (*TOX1*) were closely linked in the first genetic map (Cumagun *et al.,* 2004; Jurgenson *et al.,* 2002). The *MGV1* gene is associated with all of the phenotypes known for these loci (Hou *et al.,* 2002). We hypothesized that mutations in *MGV1* gene could result in the different phenotypes observed in Z-3639 and R-5470. The *MGV1* gene was sequenced from R-5470, Z-3639 and PH-1. For functional studies, chimeric structures were made to exchange the regions upstream of the *MGV1* gene between R-5470 and Z-3639, and fungal transformation was done by a polyethyleneglycol (PEG)-mediated method with protoplast as the recipients (Lee *et al.,* 2002; Figure 2.1).

Comparison between estimated and actual size of genetic markers: The size of the genetic markers in the gels was estimated by comparisons with the Low Mass Ladder (Life Technologies, Bethesda, MD) (Jurgenson *et al.,* 2002). We compared the estimated sizes to sizes of sequences from clones which contain the adaptor sequence used for AFLPs.

DNA isolation: DNA was extracted with a cetyltrimethyl ammonium bromide (CTAB) procedure (Leslie and Summerell, 2006). DNA concentration was determined with a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and adjusted to 20 ng/µl for PCR reactions.

Southern Hybridization: Standard procedures were used for restriction enzyme digestions, agarose gel electrophoresis and Southern hybridizations (Sambrook *et al.,* 1989). 10 µg of DNA was digested completely with restriction enzymes and separated by agarose gel electrophoresis. Fractionated DNA was transferred to Hybond N+ nylon membranes (Amersham Biosciences, Pittsburgh, PA) by capillary transfer under alkaline conditions (0.4 N NaOH, 1 M NaCl). The membranes were hybridized at 60°C with the AlkPhos Direct Labelling and Detection System (Amersham Biosciences) per the manufacturer's recommendations.

Results

Sequencing and Alignment: One hundred and twenty-nine AFLP bands were isolated and sequenced from strains Z-3639, R-5470, and PH-1. Thirty-one were sequenced directly. Of the ninety-eight markers cloned from AFLP bands, seventy were represented by three matching sub-clones and twenty-eight were represented by two or three different sub-clones. The virtual AFLPs were used to choose the correct clone from amongst each of these twenty-eight sets of fragments by comparing the sequence of the clone with bands predicted by the virtual AFLP analysis. For example, the marker band designated EAAMAA0315J from R-5470 was not observed for either Z-3639 or PH-1. This marker band yielded two sub-clones, one of which was 282 bp in length and the other was 283 bp. The virtual AFLP based on the PH-1 genome did not contain the 283 bp-fragment but did contain the 282 bp-fragment. Thus the 283 bp-clone was associated with EAAMAA0315J.

One hundred and twenty-nine AFLP sequences were blasted on the first genome assembly. One hundred and nineteen AFLP sequences had hits on the genome assembly. Two AFLP sequences from R-5470 were in the excluded reads of the *F. graminearum* sequencing project, which includes highly conserved repetitive sequence and ribosomal RNA genes. Eight AFLP sequences had no hits. PCR amplification with three additional bases showed that 11 of the 119 AFLP bands were monomorphic on the parents, and that the wrong AFLP band probably had been cloned.

The 108 AFLP markers and seven structural genes were used to align the genetic map with the genome assembly. LG1, LG7, LG8 and LG9 aligned with SC2 and SC5; LG2 aligned with SC3, SC8 and SC9; LG3 aligned with SC4 and SC6; and LG4, LG5 and LG6 aligned with SC1 and SC7 (Figure 2.2). These alignments revealed that several of the linkage groups were

overlapping and could be combined. The nine linkage groups identified in the first genetic map (Jurgenson *et al.*, 2002) were reanalyzed and reduced to six linkage groups. Linkage groups 1, 7, and 8 were merged into LG1, and LG 5 and 6 were merged into LG5 in the new genetic map (Figure 2.2). The total size of the linkage map was reduced from 1286 cM to 1140 cM. The alignment grouped the linkage groups and supercontigs into four sets, which is consistent with the hypothesis that there are four independent chromosomes in *G. zeae*. The estimated sizes of the chromosomes are 11.54, 8.57, 7.66, and 7.86 Mb (Table 2.2). Of the 108 remaining AFLP sequences, seven (one from PH-1, two from Z-3639, and four from R-5470) were present in part in the genome assembly. The other 101 markers were present in their entirety in the genome assembly with 83 to 100% identity (Table 2.1).

Markers without hits on the genome database: Eight AFLP markers (4 from Z-3639 and 4 from R-5470) were not found in either the genome assembly or the excluded reads in the genomic database, and did not have significant hits in either a blastn or a blastx search against sequences of other organisms in the NCBI database (http://www. ncbi.nlm.nih.gov/).

Two of these markers (EATMCA0383J and EAAMTG0323J) were located at the end of linkage group 6. EAAMAG0123K and EAAMCA0680J were located at the ends of linkage groups 7 and 9, respectively. The other four markers (ETGMGA0314K, EAAMGC0142J, EATMGA0624K, and EAAMAT0513K) were closely linked on the end of linkage group 2. In Southern hybridizations, all of the markers were present in R-5470, Z-3639 and PH-1.

Sequence similarity of genetic markers with the genome assembly: Fifty-five of the AFLP markers sequenced (total size: 12.63 kb) originated from R-5470. Fifty-one of the 55 had sequence similarity of 83 to 100% with ~ 96% average sequence similarity. Four AFLP markers were not found in the genomic sequence (Table 2.3). Forty-one of 45 AFLP sequences (total

size: 11.33 kb) originating from Z-3639 had sequence similarity from 87 to 100% with ~97% average sequence similarity, but four AFLP markers were not found in the PH-1 genome sequence (Table 2.3). All eighteen markers originating from PH-1 were identical to the genome sequence from that strain except for a few base pairs (Table 2.3).

Localization of *nit1* and *nit3*: *nit-3* of *N. crassa* had a hit on FG01947 in the *G. zeae* genome database (E-value of 0.0). FG01947 is located on contig 1.104, which is consistent with the location for *nit1* suggested by Jurgenson *et al.* (2002). *nit-4* of *N. crassa* had a hit on FG02799 (E-value of 0.0), but the location of FG02799 is on a different chromosome from the expectation by Jurgenson *et al.* (2002). Five other genes (FG03929, FG04666, FG06481, FG07927, and FG07079) with low E-values were not linked to the location of the *nit3* locus suggested by Jurgenson *et al.* (2002). The *G. zeae* genome sequence has 65 homologs of *nit-4* of *N. crassa* below an E-value of 1e-3. FG04901 (contig 1.199), which is consistent with the location suggested by Jurgenson *et al.* (2002), is ranked as the 16th best match with an E-value of 1e-12.

Analysis of *MGV1*: The R-5470 amino acid sequence of *MGV1* was identical to that of PH-1 and differed by only a single amino acid from the *MGV1* sequence in Z-3639. The upstream region of R-5470 *MGV1* contains a 10 bp deletion that might be responsible for the unusual phenotype observed in this strain. However, when the upstream region of Z-3639 *MGV1* was replaced with that of R-5470 and vice versa (Figure 2.1), no change in phenotype in either strain was observed.

Comparison between estimated size and actual size of genetic markers: The actual size of genetic markers was compared to the estimated size reported in the genetic map (Figure 2.3). The correlation equation was $Y = 0.91 X + 28.84$, and the R^2 was 0.98. The correlation

was high but the accuracy had a little variation. Smaller fragments (approximately < 200 bp) were under-estimated, middle size fragments (200 bp - 400 bp) were properly estimated, and larger fragments (400 bp - 700 bp) were over-estimated. Fragments larger than 700 bp were not reliably sized, and the variance in size estimation for these fragments was large.

Discussion

We aligned the genetic map with the first assembly of the genomic sequence of *G. zeae* strain PH-1 (lineage 7) that was released by The Broad Institute (Cambridge, MA). We used 7 sequenced structural genes and 108 sequenced AFLP markers from all nine linkage groups (LG) of the genetic map and aligned them with nine supercontigs (SC) of the genomic sequence. LG1, LG7, LG8 and LG9 aligned with SC2 and SC5; LG2 aligned with SC3, SC8 and SC9; LG 3 aligned with SC4 and SC6; and LG4, LG5 and LG6 aligned with SC1 and SC7. The nine linkage groups in the previous genetic map were reduced to six linkage groups, and the total size of genetic map decreased from 1286 to 1140cM. Approximately 99% of the sequence was anchored to the genetic map, indicating the high quality of the sequence assembly and the relative completeness and validity of the genetic map. The alignments grouped the linkage groups and supercontigs into four independent sets, which is consistent with the hypothesis that there are four chromosomes in this fungus.

The alignment between the genetic map and the genome assembly is consistent with the results of Gale *et al.* (2005). The results are also consistent with the previous cytological research (Howson *et al.,* 1963; Taga *et al.,* 2003). Taga *et al.* (2003) showed that *G. zeae, F. pseudograminearum, F. culmorum, F. cerealis,* and *F. lunulosporum* all have four large chromosomes which is amongst the lowest for filamentous fungi. *Mycosphaerella graminicola* has 20 chromosomes, 12 of which were < 2 Mb in length. *F. verticillioides* (Xu *et al.,* 1995), *M.*

grisea (Skinner *et al.*, 1993), and *N. crassa* (Orbach *et al.*, 1988) contain 12, 7, and 7 chromosomes, respectively. *F. oxysporum* has a range from 9 to 14 chromosomes which is associated with vegetative compatibility groups (Boehm *et al.*, 1994).

Eight genetic markers from the cross were not found in the genome assembly (Table 2.1). Four (one from R5470 and three from Z3639) of these markers occur in a SC 3 region at the end of LG2. Based on Southern hybridizations, PH-1 also contains these fragments, which suggests that the assembled genome may not contain this region. In this study 1140 cM of the genetic map was aligned with 35.63 Mb of the genomic assembly and at least 155 kb corresponding to 5 cM could be missing in the genome assembly of SC3. The other four markers (6A, 6E, 7B, and 9D) not found in the genome sequence were located in regions that correspond to the ends of supercontigs, suggesting that the genome database may be missing terminal regions of SC1, SC5, and SC2.

Seven AFLP markers had partial hits in the genome assembly (Table 2.1). One of them (EATMCG0165KH) was isolated from PH-1, suggesting that the genome assembly may still be incomplete around this fragment. Six other markers (two from Z-3639 and four from R-5470) were located in a nonsyntenous region (Figure 2.2), which could be explained by a heterozygous chromosomal rearrangement or transposition.

Severe segregation distortion on three of five linkage groups in the first genetic map (Jurgenson *et al.*, 2002) could be explained by the selection method used to obtain the progeny. The genetic map was constructed by crossing complementary *nit* mutants of Z-3639 and R-5470. The progeny analyzed were selected for the wild type alleles at both *nit* loci and segregation distortion of some markers in progeny can be expected. Jurgenson *et al.* (2002) suggested *nit1* and *nit3* might be located at 4AC and 2C, respectively. *nit1* is located on contig 1.104 of the

genomic sequence, which is consistent with expectation of Jurgenson *et al*. (2002). Segregation distortion around 4AC in linkage group 4 could result from selection for the wild type allele at *nit1*. Two other linkage groups (LG5 and LG6) showing segregation distortion were aligned with the same chromosome as with LG4 and the distortion could be affected by the *nit* selection process.

Localization of *nit3* in the *G. zeae* genome remains an open question. Jurgenson *et al.* (2002) predicted that this gene was located near locus 2C of linkage group 2 because markers around 2C were extremely skewed to R-5470. Based on a search of the *G. zeae* genomic sequence with the *nit-4* gene of *N. crassa*, the best match (FG02799) is located on contig 1.141 which is aligned with LG1. FG02799 probably is not *nit3* because there is no distortion in the region where this sequence currently is placed. If FG02799 is *nit3*, then either the genetic map or physical assembly of the genome is wrong. This is unlikely because the region of contig 1.141 was properly aligned in both analyses and the map of Gale et al. (2005). Alternatively, another sequence might represent *nit3*. The best possible candidate based on the 2C locus predicted by Jurgenson *et al.* (2002) is FG04901 (contig 1.199), which is the 16th best match (E-value of 1e-12) for *nit-4* of *N. crassa*. As *nit3* is a regulatory gene, the constraints on sequence identity may be less than those on a gene such as *nit1* that encodes a portion of a functional enzyme. Alternatively, the *nit3* region could be missing from the genome assembly. Four of the unrepresented markers are closely linked to locus 2C, which increases this possibility.

Segregation distortion also could result from multiple sequences that fortuitously were the same size, as noted by Jurgenson *et al.* (2002). In silico AFLP analysis found that one AFLP band may contain several fragments with different sequences. For example, we found one locus, ECCMCG0177J with the band originating from R-5470, that localized to the center of LG1.

This band contained two DNA fragments of the same size (168 bp), one of which is found on contig 1.111 of the genome assembly (LG4 on the genetic map) and the other on contig 1.450 (LG2 on the genetic map). In silico AFLP did not identify either fragment in PH-1. Since the two fragments were unlinked, we expected 75% of the progeny to have an R-5470 marker pattern with segregation distortion. However, the segregation ratio observed was 1: 1. This seeming contradiction is resolved since the sequence located on contig 1.111 is tightly linked to *nit1* and the other sequence is located on a chromosome with *nit3*. Thus, this genetic marker was mapped incorrectly on LG1 due to the selection for wild-type *nit1* and *nit3* alleles. Such mapping errors appear to be relatively few.

There are portions of LG2:chromosome 3 and LG4:chromosome 1 where the order on the map does not correspond to the proposed physical sequence. These anomalies could result from mapping errors or errant assembly of the genomic sequence. These alignment problems are not present in the map of Gale *et al*. (2005). An alternative explanation suggested by Jurgenson *et al.* (2002) was that large heterozygous inversions had occurred in these regions in their cross, specifically from 2F to 2AJ and from 4C to 4P. The alignment problems for both LG2 (2T to 2AE) and LG4 (4C to 4K) are contained within the regions predicted to be inverted by Jurgenson *et al.* (2002).

Jurgenson *et al*. (2002) reported recombination suppression on four linkage groups. Three of these linkage groups (LG7, 8, and 9) align with chromosome 2, which carries mating type (contig 1.358). Recombination suppression has been reported for chromosome 1 of the pseudohomothallic species *Neurospora tetrasperma* (Gallegos *et al.,* 2000), where mating type is included in a large genomic region that fails to pair properly during meiosis (Gallegos *et al.,* 2000; Fraser and Heitman, 2004). Mating type loci-related recombination suppression also has

been reported in the basidiomycetes *Ustilago hordei* (Lee *et al.,* 1999) and *Cryptococcus neoformans* (Lengeler *et al.,* 2002). If *G. zeae* has a chromosomal rearrangement around the mating type gene, then recombination suppression such as that observed in *N. tetrasperma* is to be expected.

Another possible explanation for the recombination suppression could be high levels of polymorphism on chromosome 2 of *G. zeae*. In *N. tetrasperma*, chromosome 1 has high levels of heterogeneity that are related to recombination suppression (Merino *et al.,* 1996; Gallegos *et al.,* 2000). In the genetic map of Jurgenson *et al.* (2002), the number of polymorphic markers per Mb of physical sequence was 25, 38, 26, and 27 for chromosome 1, 2, 3, and 4, respectively. Also in the map by Gale *et al.* (2005), the number was 6.0, 8.6, 5.1, and 6.3. In the both maps, the number of polymorphic markers is higher for chromosome 2 than for the other chromosomes. Such an increase in heterogeneity could help to explain the recombination suppression observed in the first map (Jurgenson *et al.,* 2002).

O'Donnell *et al.* (2004) separated different lineages of *G. zeae* into nine different species based on the genealogical concordance phylogenetic species recognition concept. They misinterpreted several properties of the Jurgenson *et al.* (2002) genetic map as support for their hypothesis that the parents of that cross belong to two different species: *F. graminearum* (Z-3639) and *F. asiaticum* (R-5470)

They claimed that high levels of segregation distortion are indicative of large-scale chromosomal rearrangements between parental strains. However, the selection method used to obtain the mapping population explains a large proportion of the observed segregation distortion. Chromosomal rearrangements are not uncommon within a fungal species, e.g. *Neurospora* (reviewed by Perkins, 1997), although there has been no systematic search for them in

filamentous fungal plant pathogens. There are other explanations for segregation distortions other than chromosomal rearrangements, e.g. the spore killers known in *F. verticillioides* and *F. subglutinans* (Sinhu, 1984). The putative chromosome rearrangements in the Jurgenson *et al.* cross are associated with segregation distortion only due to their linkage to the *nit* loci.

O'Donnell *et al.* (2004) also confused linkage groups with chromosomes. Linkage groups always are ≥ the number of chromosomes. In both this alignment and that of Gale *et al.* (2005), the number of linkage groups is > 4. These linkage groups are assigned to chromosomes through associations with common supercontigs. Only by using information from both the genetic maps and the physical sequence can the number of chromosomes in *G. zeae* be determined to be four.

A third problem claimed by O'Donnell *et al*. is that the gene order established by the linkage relationships was inconsistent with that for the PH-1 genome sequence. As shown here, there are only two regions in which the genome sequence and Jurgenson *et al.* map are not well aligned. These two regions are the location of putative inversions that were predicted on the basis of unusual recombination patterns in those regions. A few other loci, e.g. 6F, elongation factor, and 3B, appear relatively out of place. Such misalignments are not uncommon for *N. crassa*, *A. nidulans*, and *M. grisea* (http://www.broad.mit.edu/annotation/genome/), in which the genomic sequence and genetic map were independently derived. Gale *et al.* (2005) used both recombination frequency and the genomic sequence to create their genetic map.

Finally, O'Donnell *et al.* claim that a significant number of orthologs are not shared by parental strains of the Jurgenson *et al.* (2002) mapping cross. However, half of the AFLP markers not found in the PH-1 sequence database were from Z-3639. In addition, all eight missing markers were detected in PH-1 plus both parents by Southern hybridizations. Thus the

problem is with gaps in the PH-1 sequence database rather than unshared orthologs. The sequence similarity of the AFLP markers from the two parents with PH-1 also were similar, with an average of 96% sequence similarity between 55 markers from R-5470 and the PH-1 genome sequence and 97% sequence similarity between 45 markers from Z-3639 and PH-1 (Table 2.3). The good alignment of the Jurgenson *et al.* (2002) map with the physical sequence of PH-1 and the overall similarities amongst PH-1, Z-3639, and R-5470 do not support the hypothesis that these three strains belong to distinct species.

One objective of this research was to evaluate *MGV1* as a candidate for *PATH1* identified by Cumagun *et al.* (2004) and several closely linked loci for perithecial production (*PER1*), red pigmentation (*PIG1*), and the amount of toxin produced (*TOX1*). Although the knock-out phenotype of *MGV1* and chromosomal location was a good match, we were unable to demonstrate that *MGV1* accounts for the mutation in R-5470.

There are still significant areas to explore in the genetic map of *G. zeae*. For example, what is the mechanism that results in multiple linkage groups on a single chromosome even when both of the available genetic maps are relatively well saturated? Similarly, do the centromeres and telomeres of these relatively large chromosomes have any unusual properties? Given the regions identified as missing from the PH-1 sequence, are there significant portions of the genome that have somehow been excluded from the available sequence? Finally, as sequencing becomes less expensive, more strains from more lineages can be sequenced. In the future, comparative genomics will elucidate the frequency and biological significance of chromosomal rearrangements in the *F. graminearum* clade.

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Table 2.1 Markers sequenced in this study

Marker name[a]	Location on genetic map	Origin			Size (bp)	Supercontig	Contig
		R[b]	Z[c]	P[d]			
	Chromosome 1						
EATMCA0383J	**6A**	**+**	**-**	**-**	**291**	**no hits**	
ECCMCG0580K	6	-	+	*+	422	1	4
EAAMTG0323J	**6E**	**+**	**-**	**-**	**266**	**no hits**	
EATMCG0731K	6E	-	*+	+	489	1	6
EAAMAA0090J	5E	+	-	-	65	1	10
EAAMGC0435J	5E	+	-	-	392	1	13
EAAMCA0404J	5E	+	-	-	373	1	15
EAAMAC0295K	6F	-	*+	+	239	1	10
EAAMCA0557J	6F	+	-	-	466	1	20
EAAMAA0717J	6G	+	-	-	610	1	25
EGAMAT0721J	5D	+	-	-	614	1	32
EAAMCA0246J	5G(H/L)	-	*+	+	201	1	37
EAAMAG0388K	5H	-	*+	+	349	1	45
EAAMAT0097J	5J	+	-	-	62	no hits	Ex[f]
EAAMGC0111K	5J	-	*+	+	81	1	52
EATMCA0352J	6M	+	-	-	223	1	52
ECCMGT0362K	6I	-	+	*+	243	1	53
EATMCG0165K(H)[e]	5K	-	+	*+	84	1	69
EAAMCC0116J	6K	+	-	-	82	1	76
EATMGA0374J	4	+	-	-	327	1	111
ECCMGT0614K	4AC	-	+	-	505	1	112
EATMGA0676K	4AA	-	+	*+	503	1	114
EAAMAC0217J	4T	+	-	-	183	1	122
EAAMAT0192K	4P	-	*+	+	160	7	420
MGV1	*MGV1*					7	425
EATMCA0305J	4O	+	-	-	195	7	426

EGAMTA0339J[e]	4K	+	-	-	280	7	446
ECCMGT0582K	4	-	+	-	478	7	446
EATMCA0199J	4I	+	-	-	94	7	441
EGAMAT0378K[e]	4F	-	+	-	361	7	436
EGAMAA0344J	4D	+	-	-	289	7	433
EAAMGC0119K	4C	-	*+	+	87	7	433
	Chromosome 2						
ECCMGT0714J	8	+	-	-	461	5	323
EAAMAC0350K	8G	-	+	-	294	5	323
EAAMCA0491J[e]	7	+	-	-	422	5	323
EAAMAG0158K	7P	-	*+	+	121	5	323
EAAMGC0320J	7L	+	-	-	263	5	329
Elongation factor	Elongation factor					5	355
ECCMGT0315J	7	+	-	-	257	5	348
EAAMAG0123K	**7B**	**-**	**+**	**-**	**90**	**No hits**	
EAAMAA0675K[e]	7B	-	+	-	559	5	358
EGAMTC0740K	8C	-	+	*+	646	5	358
EGAMAT0181J	8B	+	-	-	144	5	353
EAAMCC0237J	7A(H/L)	-	*+	+	196	5	360
EAAMTG0298J	1A	+	-	-	240	2	143
ECCMCG0193K	1	-	+	*+	163	2	145
EAAMAT0263K	1D	-	*+	+	214	2	145
EATMCG0329J	1E	+	-	-	271	2	148
ECCMGT0168J	1F	+	-	-	74	2	148
Reductase	Reductase					2	150
EAAMAC0392K	1I	-	*+	+	355	2	153
EATMCA0327K	1I	-	+	*+	266	2	153
EAAMAA0347J	1L	+	-	-	290	2	158
EATMGA0657J	1	+	-	-	480	2	159
TRI5	*TRI5*					2	159
EGAMAA0177K	1	-	+	-	139	2	161

EAAMCA0294K	1P	-	*+	+	237	2	162
EGAMTC0600J	1	+	-	-	496	2	162
EAAMAA0377K	1	-	*+	+	329	2	168
EAAMTG0185K	1AD	-	+	-	151	2	167
EAAMGC0387K	1	-	*+	+	354	2	170
EAAMAG0727K	1Z	-	*+	+	624	2	179
EAAMCA0283J	1Z	+	-	-	230	2	179
EATMCA0194K	1V	-	+	*+	163	2	181
ECCMGT0347J	9N	+	-	-	229	no hits	Ex
EGAMAT0360J	9N	+	-	-	307	2	187
ECCMAT0288J	9M	+	-	-	180	2	190
EGAMTC0432K	9L	-	+	-	384	2	192
ECCMGT0446J	9K	+	-	-	332	2	192
EAAMCA0680J	**9D**	**+**	**-**	**-**	**578**	**no hits**	
EAAMAT0100K	9C	-	*+	+	72	2	194
EATMCA0149K	9B	-	+	*+	107	2	194
	Chromosome 3						
ETGMGA0314K	**2H**	**-**	**+**	**-**	**259**	**no hits**	
EAAMGC0142J	**2H**	**+**	**-**	**-**	**107**	**no hits**	
EATMGA0624K	**2I**	**-**	**+**	**-**	**508**	**no hits**	
EAAMAT0513K	**2J**	**-**	**+**	**-**	**430**	**no hits**	
EATMCG0537K	2J	-	+	*+	445	3	197
EATMCA0309K	2N	-	*+	+	250	3	234
EAAMAA0185J	2O	+	-	-	154	3	237
EAAMCA0307K	2	-	*+	+	249	3	240
EATMGA0797K	2R	-	+	*+	660	3	254
EGAMAT0800K	2T	-	+	*+	751	8	451
TRI15	*TRI15*					8	457
ECCMCG0652K	2V	-	+	-	536	8	460
EATMCA0602K	2	-	+	-	492	9	463
EAAMTG0174J	2W	+	-	-	133	9	463

ETGMGA0127K	2	-	+	-	93	9	464
EAAMAG0314K	2X	-	+	*+	253	9	465
ECCMAT0417J	2AB	+	-	-	322	9	469
EGAMTC0386K	2AB	-	+	-	289	9	470
EAAMGC0297K	2	-	*+	+	241	8	459
EATMGA0279K	2	-	+	*+	164	8	459
EAAMAT0323J	2AE	+	-	-	264	8	459
	Chromosome 4						
ECCMAT0358J	3A	+	-	-	244	6	370
EATMCA0377J[e]	3B	+	-	-	279	4	320
EAAMCA0271J	3	+	-	-	221	6	373
EAAMAC0186K	3	-	*+	+	153	6	377
EAAMAA0119K	3F	-	*+	+	96	6	373
ECCMCG0418J	3H	+	-	-	330	6	373
EATMGA0455J	3H	+	-	-	397	6	382
ECCMAT0528J	3J	+	-	-	441	6	382
ETGMGA0145J	3J	+	-	-	107	6	384
EAAMCC0239J	3K	+	-	-	196	6	395
ECCMAT0278K	3K	-	+	*+	150	6	398
ECCMGT0373K	3K	-	+	*+	270	6	401
TRI101	*TRI101*					4	321
Phosphate permease	Phosphate per					4	321
ECCMAT0117J	3O	+	-	-	69	4	320
ETGMGA0677K	3S	-	+	-	570	4	318
ECCMCG0627K	3S	-	+	*+	520	4	318
ECCMGT0251K	3T	-	+	*+	149	4	318
EAAMAC0786K	3U	-	*+	+	734	4	318
ECCMCG0544J	3	+	-	-	448	4	318
EAAMAT0188J	3X	+	-	-	155	4	315
EAAMAT0270K	3	-	+	-	217	4	315
EATMCA0212J	3Z	+	-	-	102	4	316

EATMGA0123J[e]	3	+	-	-	68	4	316
EAAMTG0506K	3	-	+	-	431	4	310
EAAMAG0216K	3AC	-	*+	+	183	4	313
EATMGA0170K	3AF	-	+	-	133	4	308
EAAMCA0327J	3AG	+	-	-	270	4	301
EAAMCC0186K	3AI	-	*+	+	151	4	297
EATMCG0704J	3AL	+	-	-	547	4	275
EATMCA0196J	3AM	+	-	-	167	4	266
ECCMGT0482K	3	-	*+	+	413	4	259

[a] The markers were named in the previous work (Jurgenson *et al.*, 2002) using the nomenclature E_M_0000_, where E_ denotes the *EcoRI* primer with the two additional selective nucleotides, M_ denotes the *MseI* primer with the two additional selective nucleotides, the four-digit number is an estimate of the size of the band in base pairs, and the final blank is either "J" or "K" and denotes the parent that was the source of the "band present" allele or was the source of the larger band of a size-different polymorphism.

[b, c, and d] markers from R-5470, Z-3639, and PH-1, respectively.

* the origin of sequenced marker.

[e] sequence with partial hit to the genome database.

[f] hits on the excluded reads of the genome database.

Table 2.2 Chromosomes of *G. zeae*

Chromosome number[a]	Supercontig (SC)	Linkage group (LG)	Size		No. of markers[d]
			cM[b]	Mb[c]	
1	1, 7	4, 5	329	11.54	29
2	2, 5	1, 9	368	8.57	37
3	3, 8, 9	2	238	7.66[e]	18
4	4, 6	3	205	7.86	31
Total	9	6	1140	35.63	115

[a] Chromosome number was ordered by Gale *et al.* (2005).

[b] Centimorgan.

[c] Megabase pairs.

[d] The number of genetic markers used for alignment between linkage groups and supercontigs.

[e] This estimate does not include 155 kb of sequence estimated to be missing from the SC3 genomic assembly.

Table 2.3 Sequence identity of genetic markers with the *G. zeae* genome assembly

Origin[a]	No. of markers[b] (No. of no hit-markers[c])	Total size of markers (kb)	Identity (%)[d]		
			Minimum	Maximum	Average
R-5470	55 (4)	12.63	83	100	95.82
Z-3639	45 (4)	11.33	87	100	97.19
PH-1	18 (0)	5.90	99	100	99.73

[a] The origin of markers; R-5470 and Z-3639 were used for the genetic map, and PH-1 was used for genomic sequencing.

[b] The number of markers sequenced.

[c] The number of markers which do not have hits on PH-1 genomic sequence.

[d] Identity based on blastn (nucleotide to nucleotide) to PH-1 genomic sequence.

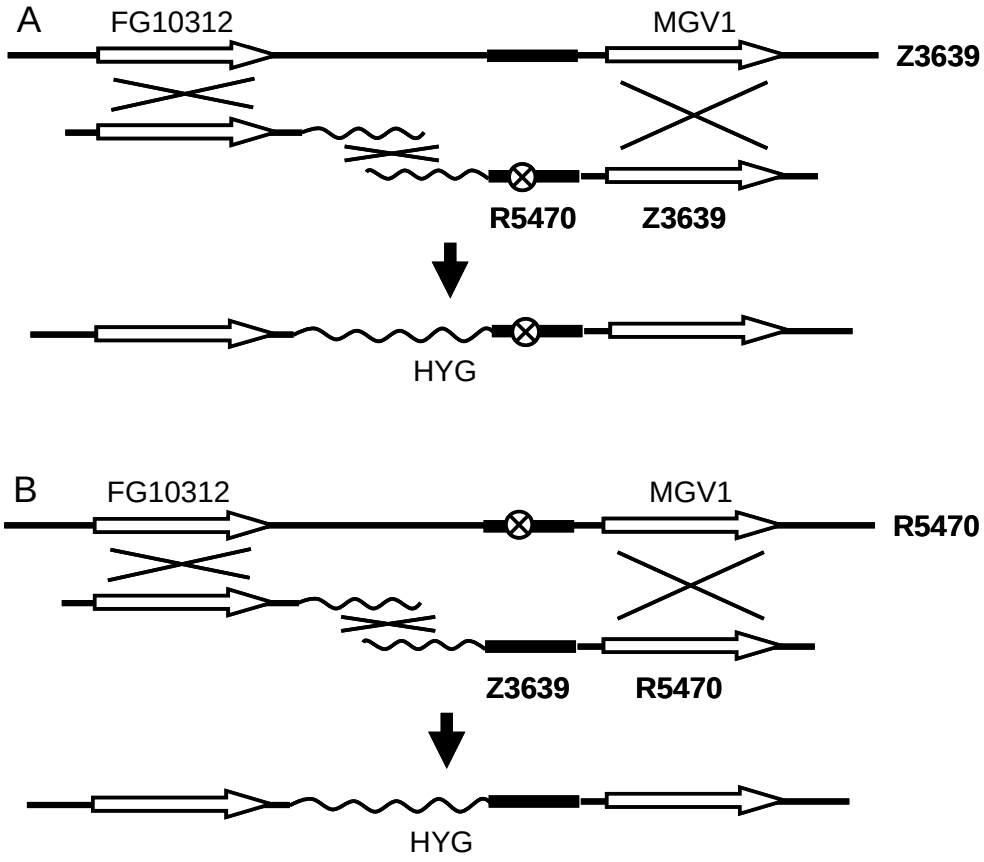

Figure 2.1 Diagram to switch the upstream region of *MGV1* gene between Z-3639 and R-5470. There is a 10-bp deletion in the region upstream of R-5470 *MGV1* gene. (A) The region upstream of R-5470 was amplified for fusing with the *MGV1* gene of Z-3639. This chimeric structure was transformed to Z-3639 strain. (B) The upstream region of Z-3639 was fused to the R-5470 *MGV1* gene and transformed into R-5470 strain.

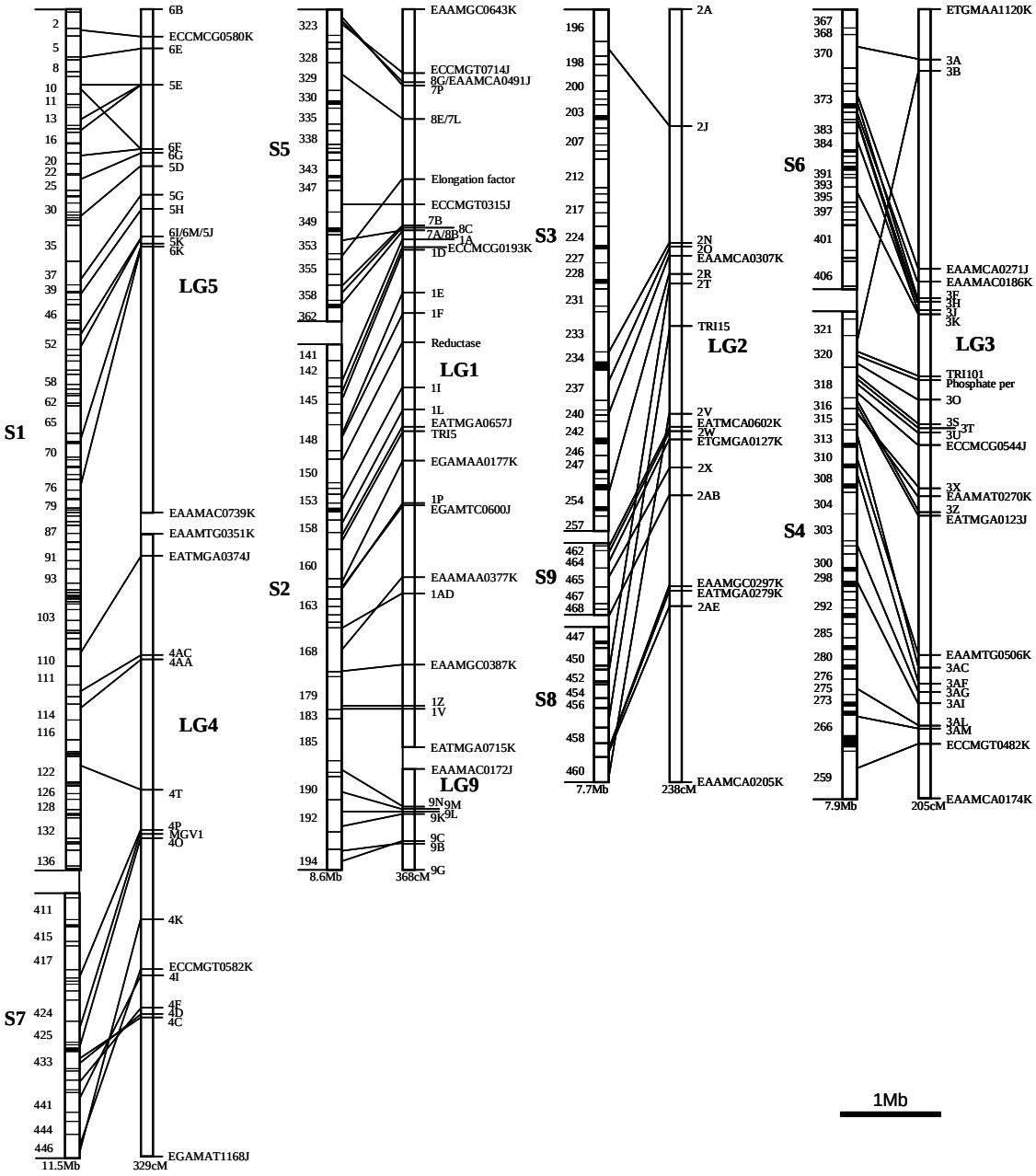

Figure 2.2 Alignment between physical (left) and genetic (right) maps. The numbers on the left side of the physical map indicate contigs. The number of each supercontig on the physical map is designated S1-S9. The number of each linkage group on the genetic map is designated LG1-LG9.

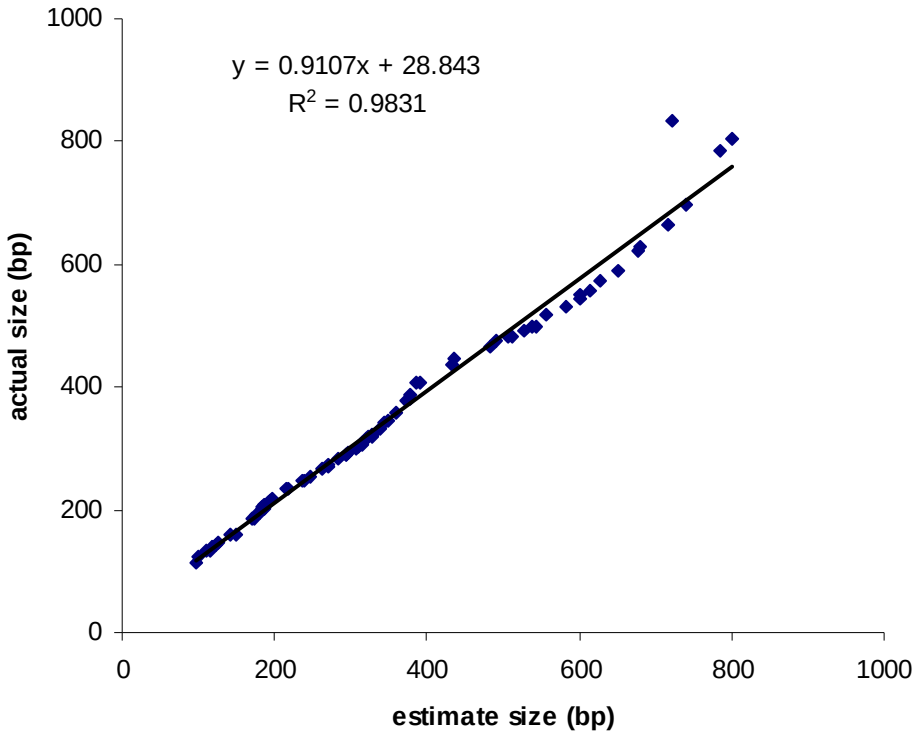

Figure 2.3 Comparison between estimated size (Jurgenson *et al*., 2002) and actual size of genetic markers determined by sequencing.

Supplementary Material

Sequences used to align the genetic map and the physical assembly:

> 3AL (EATMCG0704J)
AGACCTAGTTCATCGTAGCATTCGATCATAAACgCTGGACTTGGGGTCAATGTCATGACTTGTCAGGTTGGCAGTCGAATAtATAAT
GTCTACAGGAGAAGTGTCGGTATTGACgGGTAGGTTGATCACGGAAGAGAGGCATCGCACACAAACACGCTGGGCAAGAATCAC
CATGTTAGTTACCTGTAGCGCAATATGTAAAGTAGCATGAGAAACTCACCCTTTCACCTCCGTTGCTGGCAGAAGCTGGTGCATCG
GTACCTGGCACTATGCCTTTGCCTCCCTGCTTTATGAAGCTCATTTTTTCTGGCAAAGAAGATTCCTCAGGACTGTTGGAACGGCTG
AAATCTAATGAAGAGATGGTCGGTGCCCCGGGGCTTGGCGTGCTGGGTTGACTTGCTACAGCATTCGATCGCCTGGCAAGGTAGA
AAAGCTTGTCAAGGACAGAAAACCGTGGGAATCTCGGAGAACGGATGGGAGGTTGTGCTTGCACGCGCGCGAGGTCACACTTCTT
CTGCTCAGCGCTGATACGATCGTTCTCAGCGTTA

> 6E (EATMCG0731K)
CATACCACCAGCCCATAGCAACCATAGCCGTGCTCCTTGAGNTTTGGCAAGTTGTGTCTTGACCAATCCCACGGGGGNACNAGCG
CAAATGGGCTTTTGCGTATCAGGTATCAAGAAAGTCGACCAAAAACAGCGGCACATTGTCCTNCCAGGGAATGCGCCTAAAGGTA
GAAAGGTCTGGTATCGCTAGAAGATCATGGTAACCGTTCTCGGCATTTGAGATCTTGAGCTCGCNGACATATGTGCGGTCGTGCAT
GTCCCAACCAAAGATGGACGGAGCAGAAACCGAAGCCGGCTTCTGCGATGGACAGGAACTTTTTCTTGGAGACGAGCTTTCCGCG
NAAGGATGCCGTCGACCGTCGACGCCTGCGAGTTTGACCATGTTGTCATTCTCGAGGACTTGGGCGAGATTGTCAGACGGTTGAC
CGGCTTTGCTGAGGAAAGCCATNTTCTGGGAGTATGTGAGGAGAGCTACTGAGGCTTCCAGAGGAGCAGT

> 5K (EATMCG0165K)
TCCCAATGNACAAGCGATGTCTGTGTAATACCATTGGCGTACTGCTCCATATATAGAGCAAGTGAGGTGCAGAGTTTGTTTCCGTT
ACCTCAGGACTCATAC

> EATMGA0657J
GAATGCTTGAAGATTGTCGAGACACCTGTATCTTCCTGCATCTTTGTCGACGACAAGGCAGAAAACGTTGTTGCCGCGCAGACCCT
TGGTTTCAAGAGTATCGTATTCAGTGATACTGACAATCTGGTCAGAGACCTTCACAACCTCCTTGGTGACCCTGTTGCACGTGCAA
ATTCGTTTCTTGAAGGCAAGTTGAAGAGCACTGGTAGCATATTGTTGGACAATTACTCTCAATTTATCGTGCTTCAAAACACGGGA
ATCCGGTATGTCTGTCAGCATCTCTCTTATCAGGCTGTTGACTAATTCTTCTAGGGATCTGATCATGCCAGAGAGTGACAGTAGGG
ACTATTTCCAGCAAGGATCGCCGATACTGACGACCACGGTCCATCCCGAAGGCTCTGACACCACATCTCTGGTGTTGGCAGTCTTG
GACAATGTTCCAATGGATACGAAGCTCAAGGCCAGAGATGAGATACTCTTCNCNNGGGAACTCATC

> 3H (EATMGA0455J)
GATGAGTCCTGAgTAAGAAGGTATGTCCGAGATaAGTTTGGAGGCTTGGAGCTAATATTGAGGCTCAGGATAGCCACATGATTCCC
GATTGGATATTGTTTTGTGATGGAAGCCATGTTCGAGGGATTTACATGGGATGGTCAATATCAcGAGATACTGTTCTGACAGTCTA
GATGTATCAGCTTGtACGGAGCgCTCGACTGTSGTATCGTTGACCCCTTGCAGCGATGACAATTACAGCACAACCTCCATAGGATA
CGATGATGGGTGCGTCGTTTATTGTTGCCTGTTTTGTTTGACGTtGCGTAAGGGTASaGTAGATAACCCCAGGACGACGGATSGGCT
AGACTCTGCGACTATACCACTTACAGCCAACCGGTTGCTGAGCAAAGAAGCATGTAATGATGAATTGGtACGCAGTCT

> EATMGA0123J
CGCTATGAGAGTTGATGAGCAGAATTGTGTACAAAAAGGACGTCTTGTTCGCTCATAATCATGCTCTTACTCAGGACTNATC

> 2I (EATMGA0624K)
GATGAGTCCTGaGTAAGAGATACTGCAGGGCGATCTCGTTTTGCGTGTCGAGCCGCGtTACGAGCAGTCCATTTGCCAATCATATCT
TCCAAGCCCCCCCATCAGGTGGTACAGTCGAGACGAGCTGTCAGGAATCTCGCTCCTCCAGCGAGCCACTTCACGtGTCAAATTCGT
CTGATATTGTTGCAGTTTTATCGACGTCGTCTTCCGTCCGTTCGCTCATGTCGGACATTCTTGCCAGGTCGTTCTCAGACCCCATCT
CAGGTGAWTAAGAAGAGTCTTTATCGTCTGGATTCAAATCCCAAGGAAGGGCTTTCAATGCCCATGAATGAAGGTGCTCTGAAAT
GTGATTATCTTGATCTGGCCTCGgAGTATCCACATGGCCGTATACAAAGAGGACACTCGGCTAACTGGGCGAGGACTCTTTTGCTC
ATACTGCAGAGGAGTTTCAAACGATCATCCGAGCAGACGNGANNGGATCNCATGTGGTTTTCCCAAAGNTCCTGGTTTTCCAAGA
TCTAATTGGTTACNC

> 2R (EATMGA0797K)
TTATCCGATGAAAAGAAGCACNCAGAGAAATGTAAAAAGCGAGTGGGCGGNATTACANGAGATGAAAAAGGAGAAGGAAGACG
AGCAAGATGTTTTTATACAACCTTTGTTTTATGTATGATCACCTATATTATCTATCATAGAGCTAGGGCCTCTATGTATCAACACCC
AGAGGTCAAACAAGTATCGCAGATTCATGATTGCGTATTTCTGAGGCCCAATGGTTGACTTCTTCACTGTTCGGGTCAACGTCGTC
CCTCTTCTCAAATCAGCATGCCAACGAGGCTTCAGTATGGGTGTAATGAGCTAGCTGTTACTCTCATGAGGTCATGTCGAATAGTT
AGACCGACCCATCGACTGGGTAATCGTAAGGGGTAGACGGGAAATCGCCCGTTGTCGAAAGGATGGTAAAAGTACTATCTAGCT
AATAGAGATCATAGGCCGATCAACCGGCTAAAGACTTATAGTGACTGCACTTGACTGATTCATGGTCAGCTACAGCATTGAAAGT
AACCAATGGTTATATCAAGCTGCGACAAAAATCCCTTGGATGACTGTAAGAAAATGAGTGCATGCAGAAGATCTTCCCGCGTCTC
AACAATTGGACAGTTTTCGCTCACCACTCACGCTTTTGGTTAGGCAGTTCGCGACTCGGCTTCTCT

> 4AA (EATMGA0676K)
CGTGCGTTCACATCCACCATCACCACCTTCTCCATCACTGTCAGTGCCACGGCCCGCTGcCCTCTCTGTCTCTCTCCCTCTCCATCCT
TGCCTACACGCCATGCCCACCAGCGCAGCCGTTTTTGCCATGCTGGCGTGTCTCATTCGACAACTTCTCAATCATCACATTCTTATT
GCGTCCTCCTCCCGATGCCCTTCCAAGCCGCAGCAGAGCCGCAGAATCGGCTTTCCGCAGAATCGGCTTTTGGCCAGGCGAATCA
AGACCTTTCTTCCACTCAACTGCGCAATTGTTACCCCGAACGCGATACGAACTACGCGATGAAATTGCCTGACCTGATCGATGGTT
CTTGCGCTGCGGGTTCTATTGCTAGAACAAAATGATTTGCGCCTTTGCTGTGCATGAAGCGAAATGCGTGAACTCCAGCTCGGATC
TCAGCAATAACGAAGAACGCAATTGAGGCAATTTGGGCCCGTTTGAAACGAAGTGGTACGTTGACAATGTCTTCNCANGGGNACT
CATC

> EATMGA0279K
CAATTCTTCTTCTTATAACTGACACGGACCCCTTTCACCTTAGGCACGCATTTCTTACAAAGATCTGGCGGAGTAAGAACCTGAGT
AAACCGCCGTGGTAAGCGCTATCCTTAGGGAAGAACCTTCCGTTCTCAACAGTATAATCCTGTAGCTGCTTCAGGTTCTTACTCAN
GGACTCATC

> 2AB (ECCMAT0417J)
GtGTCTGGAGCAAGCGTGTGCTGACTATGGGTCTTCTTGGTTCCTGATCGCAGAGGTGACATGATTTGAAAGAAATCTAGATTTCA
GACAGGGGACATCTTGTGGCACATTCAGGAACTCACTGCACTACTGAATGCTAACTGCGCCATCCGACGCAGGAACGGCAGGTAC
AGTTGTCGAAGTTTTCAGGTCTTAGAAGTTTATTCCCATCTATGTGATGTAAGTCAATTTCTCCTTCGGAACAGTTAGTAGTTCACA
TTCTGGAACGATCAGTAAAAGGTCAAGGCAACCTGTTGTTGCTATTCAAGTGCTCATATACATTTCCCNGGGGAACTCATC

> 3A (ECCMAT0358J)
CACACCTGGTCTTCTGTGCTGTTAGGGACATTGCCAGCTGTTATCGTTGAGGTTACCTGGATGTAGTGTAAAGGTCGGGTGGTGAA
TGGAGCTAGACATTCTTTATCATCTTATTGTTTTCTTGGCATATTTGATATATCCAGAGAACATGGTTTTGTACAATACTGTGGTGG
ATGAGATGCAGGATCAGGGACACTACCAAATTGAGCAGAAACAATGGTATTTGACATTTCAGGAGTTATTTATCCAGGGNACTCA
TC

> 9M (ECCMAT0288J)
TAACATCGGCGACACAAAACATTTACATCATTGGTGAGGAGTTGTAGGAAACATGCAATTTTGAAGGGGAACTACGGTACCCTAC
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CTTTTATTTATCCANGGNACTCATC

> 3O (ECCMAT0117J)
GACAATTATACGTAGCATATTCCTTCGTATTGCATTTATGAGGCTCAATATTGACCAGGNATGTGCATTTAACTCAGGACTCATC

> 3K (ECCMAT0278K)
AACCCCCCGGCCGATTATCCACAGACTCGAGGtATTTGTTGGAGATATTCATACTCACGAGTTTCTGTTGGTTATTATTCTGCACAT
GCGTTAGAGGTTACACTTGGACTTTGTTCACTGCAGtCCCTCAGATACCTTGCAAATCTTGATTTATCCAGGGACTCATC

> 3H (ECCMCG0418J)
CAAATCTTGAATGAAACAACTAACATGTGGTCCAACACAGTCACGATCTACACACAGACAGTGGAACTACCGCGCGGTAGAAGA
AAACAAGAACAGAAAGAAAGAAAGAAAGAAAAAAGATGCCTCGCAGGTTTCATCTGTAGATTGAGAGTAGGGATCGGCCCGTTG
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TCATC

> ECCMCG0580K
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GGGAAACTCNTC

> ECCMGT0714J
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CTTCAGTATCGGGTATATAGTCATCAACCGTGGAGGATACATCCTTTGGGCTAACGACGTTATCGTCATGTTCAAAGGATTGCTTG
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> 9K (ECCMGT0446J)
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GGAGCAGACGTGAACGCACAATATGACCGGTTTGGCACCGTCTTACAGATTGCCGCCCTGATGGGCCACCTGGAGATTGTGCAAC
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ACCTCATC

> 9N (ECCMGT0347J)
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ACACGTGTCCTTTCAGGTTGTTGACCAATTTGCCGAACTGTACAAATGCTTGGAACTTATCCANGGGACTCATC

> 1F (ECCMGT0168J)
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ATC

> 3K (ECCMGT0373K)
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> 6I (ECCMGT0362K)
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TC

> 3T (ECCMGT0251K)
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> 2AB (EGAMTC0386K)
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> 6A (EATMCA0383J)
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> 3B (EATMCA0377J)
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> 6M (EATMCA0352J)
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> 4O (EATMCA0305J)
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> 3Z (EATMCA0212J)
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> 4I (EATMCA0199J)
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> 1E (EATMCG0329J)
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> 2J (EATMCG0537K)
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> EATMGA0374J
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> 3AF (EATMGA0170K)
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> 3J (ECCMAT0528J)
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> ECCMCG0544J
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> 2V (ECCMCG0652K)
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> 3S (ECCMCG0627K)
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> ECCMCG0193K
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> 4AC (ECCMGT0614K)
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> ECCMGT0582K
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> ECCMGT0482K
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> 4D (EGAMAA0344J)
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> EGAMAA0177K
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> 5D (EGAMAT0721J)
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> 9N (EGAMAT0360J)
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> 8B (EGAMAT0181J)
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> 4F (EGAMAT0378K)
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> 2T (EGAMAT0800K)
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> EGAMTC0600J
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> 9L (EGAMTC0432K)
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> 8C (EGAMTC0740K)
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> 3AM (EATMCA0196J)
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> EATMCA0602K
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> 2N (EATMCA0309K)
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> 1I (EATMCA0327K)
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> 1V (EATMCA0194K)
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> 9B (EATMCA0149K)
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> 4K (EGAMTA0339J)
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> 3S (ETGMGA0677K)
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ACGCCTCCTCCCCCATGCACCGCGGAGTCGTCAACGTCCACAACTACGAGGTGTGCATCAAGGTCTGCTGGCCTGAGGAGCCTAC
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> 2H (ETGMGA0314K)
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> ETGMGA0127K
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> 3J (ETGMGA0145J)
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>5E(EAAMGC0435J)
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> 7L (EAAMGC0320J)
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> 2H (EAAMGC0142J)
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> EAAMGC0387K
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> EAAMGC0297K
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> 4C (EAAMGC0119K)
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> 5J (EAAMGC0111K)
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> 6G (EAAMAA0717J)
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> 7B (EAAMAA0675K)
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> 1L (EAAMAA0347J)
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> ECCMGT0315J
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> 2O (EAAMAA0185J)
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> 5E (EAAMAA0090J)
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> EAAMAA0377K
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> 3F (EAAMAA0119K)
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> 9D (EAAMCA0680J)
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> 6E (EAAMCA0557J)
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> EAAMCA0491J
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> 5E (EAAMCA0404J)
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> 3AG (EAAMCA0327J)
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> 1Z (EAAMCA0283J)
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> EAAMCA0271J
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> 1P (EAAMCA0294K)
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> 5G (EAAMCA0246J)
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> 1Z (EAAMAG0727K)
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> 5H (EAAMAG0388K)
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> 3AC (EAAMAG0216K)
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> 7P (EAAMAG0158K)
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> 7B (EAAMAG0123K)
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> 2X (EAAMAG0314K)
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> 2AE (EAAMAT0323J)
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> 3X (EAAMAT0188J)
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GGACTCATC

> 5J (EAAMAT0097J)
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> 2J (EAAMAT0513K)
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> EAAMAT0270K
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> 1D (EAAMAT0263K)
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> 4P (EAAMAT0192K)
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> 9C (EAAMAT0100K)
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> 1A (EAAMTG0298J)
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> 2W (EAAMTG0174J)
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> EAAMTG0506K
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> 1AD (EAAMTG0185K)
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> 4T (EAAMAC0217J)
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> 3U (EAAMAC0786K)
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> 1I (EAAMAC0392K)
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> 8G (EAAMAC0350K)
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> EAAMAC0186K
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> 7A (EAAMCC0237J)
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>Tri101
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>Phosphate permease
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>Mgv1 1.425
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CHAPTER 3 - PHEROMONE FUNCTIONS IN *GIBBERELLA ZEAE*

Abstract

In heterothallic Ascomycete fungi, mating specificity for sexual reproduction is controlled by two idiomorphs (*MAT-1* and *MAT-2*). Two sex pheromone/receptor pairs function in recognition and interaction of strains with opposite mating types: pheromone precursor genes 1 and 2 (*ppg1* and *ppg2*), whose products interact with pheromone receptor genes 2 and 1 (*pre2* and *pre1*), respectively. In the homothallic fungus *Gibberella zeae*, the *MAT* locus is arranged such that both idiomorphic alleles are adjacent to one another on the same chromosome. Our objective was to identify and characterize both the pheromone precursor genes (*ppg1* and *ppg2*) and the pheromone receptor genes (*pre1* and *pre2*). *ppg1* was expressed in germinating conidia and mature ascospores of *G. zeae*. *ppg2* expression was not detected in any cells. *pre2* was expressed in all cells. *pre1* was expressed weakly only in mature ascospores. Deletion of *ppg1* or *pre2* (*Δppg1* or *Δpre2* strains) reduced, but did not prevent, perithecium production following self-fertilization. When the *Δppg1* strains were used as females in crosses with male strains containing an intact *ppg1*, the amount of outcrossing dramatically increased. *Δppg2* and *Δpre1* mutants had no discernable change in morphological phenotype or capacity for self-fertilization. A *Δppg1*/*Δppg2* double mutant has a phenotype similar to that of the *Δppg1* single mutant, suggesting that neither pheromone is essential for either male or female fertility. *Δppg1* or *Δppg2* in combination with a partially disabled *MAT* allele did not alter either male or female fertility in ways other than those expected for the *Δppg1* or *Δppg2* mutations alone. Thus, one of

the pheromone/receptor pairs (*ppg1*/*pre2*) found in many Ascomycetes has a role in, but is not essential for, selfing or outcrossing in the homothllic *G. zeae*, whereas the other pheromone/receptor pair (*ppg2*/*pre1*) has no detectable function in sexual reproduction.

Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is the most important causal agent of Fusarium head blight (also termed scab) of wheat and barley (McMullen *et al.*, 1997), and also causes stalk rot and ear rot of maize, and crown rot of carnation (Leslie and Summerell, 2006). In addition to direct yield loss, *G. zeae* can reduce grain quality and harvested grains often are contaminated with mycotoxins such as deoxynivalenol and zearalenone (Desjardins, 2006).

G. zeae is a homothallic fungus and strains originating from a single haploid nucleus can successfully complete the sexual cycle without a mating partner. Other species in the genus *Gibberella* are heterothallic and strains in these species must cross with a strain of opposite mating type to produce perithecia, complete meiosis and produce ascospores. Sexual development in filamentous ascomycetes is controlled by a mating type locus (*MAT*; Coppin *et al.*, 1997). In most filamentous ascomycete fungi there are two idiomorphic alleles at a single locus. One allele, *MAT-1*, encodes three proteins – MAT1-1-1, MAT1-1-2, and MAT1-1-3 – while the *MAT-2* allele encodes only a single protein (Coppin *et al.*, 1997). Deletion of either the *MAT-1* or the *MAT-2* coding region in *G. zeae* results in strains that are cross-fertile as heterothallics, but that are not self-fertile as homothallics (Lee *et al.*, 2003).

Heterothallic fungi use small peptide pheromones and G-protein coupled receptors for communication during the mating process (Bölker and Kahmann, 1993; Dohlman and Thorner, 2001). The structure and sequence of the pheromones and the receptors are broadly conserved in

heterothallic Ascomycetes such as *Saccharomyces cerevisiae* (Kurjan, 1993), *Neurospora crassa* (Bobrowicz *et al*., 2002; Kim *et al*., 2002), *Cryphonectria parasitica* (Turina *et al*., 2003; Zhang *et al*., 1998), and *Magnaporthe grisea* (Shen *et al*., 1999). The pheromone and receptor expressed depend on the allele at the *MAT* locus, although neither the pheromone nor the receptor is encoded at this locus. The homothallic ascomycete *Sordaria macrospora* (Pöggeler, 2000) expresses similar pheromones, and both pheromones and their receptors are required for wild type levels of fertility (Mayrhofer *et al.*, 2006). *Aspergillus nidulans* also is homothallic and has functional pheromone receptor genes whose activity is essential for self-fertilization (Seo *et al.*, 2004).

The mechanism of sexual fertilization has not been identified for *G. zeae* or any other *Fusarium* species. *G. zeae* forms perithecia both heterothallically and homothallically under laboratory conditions (Bowden and Leslie, 1999), and laboratory crosses can be used to generate genetic maps (Jurgenson *et al.*, 2002; Gale *et al.*, 2005). Although the evidence is indirect, *G. zeae* outcrosses at a significant rate in North American field populations (Schmale *et al.*, 2006; Zeller *et al.*, 2004), and outcrossing appears to be an important part of the life cycle of this homothallic fungus. Pheromones similar to those found in other ascomycetes could have an important role in this outcrossing process in *G. zeae*.

Our objectives in this study were to identify and characterize the putative pheromone precursor genes (*ppg1* and *ppg2*) and pheromone receptor genes (*pre1* and *pre2*) in *G. zeae*, and to determine their function. We expected these genes to be homologous in structure and function to these genes in other Ascomycete fungi. We found that pheromone and receptor function and expression patterns were different in *G. zeae* than they are in other filamentous fungi, either heterothallic or homothallic.

Materials and Methods

Fungal strain: *G. zeae* wild type strain, Z3639 isolated from Kansas (Bowden and Leslie, 1992) and mutants derived from it were stored as frozen conidial suspensions in 15 % glycerol at -70°C (Table 3.1).

Identification of pheromone precursor and pheromone receptor genes of *G. zeae*: To identify the *G. zeae ppg1* gene, the sequences of *ppg1* of *S. macrospora* (Pöggeler, 2000), *ccg4* of *N. crassa* (Bobrowicz *et al.*, 2002), and *mf2-1* of *M. grisea* (Shen *et al.*, 1999) were used in a BlastP search of the *Fusarium* genome data base (http://www.broad.mit.edu/annotation/fungi/*Fusarium/*).

The other pheromone precursor (*ppg2*) is less conserved across species and is too short (less than 25 amino acids in *S. macrospora*, *N. crassa*, and *M. grisea*) to be found in a BlastP search. Instead, the microsynteny near *mf1-1* of *M. grisea* (Shen *et al.*, 1999) and *mfa-1* of *N. crassa* (Kim *et al.*, 2002) was used to identify the *ppg2* of *G. zeae*. Two putative pheromone receptor genes, *pre1* and *pre2* were identified from the database following BlastP searches with the pheromone receptor genes of *N. crassa*, *pre1* (sequence no. AJ313528) and *pre2* (sequence no. AJ313529; Pöggeler and Kuck, 2001), and *Emericella nidulans preA* (DAA01795) and *preB* (DAA01796; Dyer *et al.*, 2003).

Targeted gene deletion: *ppg1*, *ppg2*, *pre1*, and *pre2* were deleted by split marker recombination (Catlett *et al.*, 2003) with slight modifications (Figure 3.1A). Both the 5′ and 3′ flanking regions of a target gene were amplified by PCR with the F1/R2 and F3/R4 primer sets (Table 3.2) and an amplification protocol: 2 min at 94°C, followed by 30 cycles (30 sec at 94°C, 1 min at 55°C, 1 min at 72°C), and 10 min at 72°C for final extension. The PCR products were purified with the DNA Purification System (Promega, Madison, WI) as instructed by the

manufacturer. The hygromycin phosphotransferase cassette (HYG; 1.4 kb) was amplified with the HYG-1F/2R primers from pIGPAPA (Horwitz *et al.,* 1999) and purified with the same system. The HYG sequence for *ppg1* deletion was amplified from pCSN43 (Staben *et al.,* 1989) by PCR with the GNT-F1 and GNT-R4 primers. In this construct, the hygromycin resistance gene was flanked by a *trpC* promoter and *trpC* terminator and the size of amplicon is ~ 2 kb. The three amplicons were fused by PCR in a 25 µl reaction containing 1µl of purified 5′-flanking amplicon (100 ng/µl), 1 µl of 3′-flanking amplicon (100 ng/µl), 3 µl of HYG amplicon (100 ng/µl), 2 µl of dNTP (2.5 mM each), 2.5 µl of 10 × PCR buffer including $MgCl_2$, 1 unit ExTaq polymerase (Takara Bio Inc., Japan), and 15.25 µl of water. The PCR amplification conditions were 2 min at 94°C, followed by 10 cycles (30 sec at 94°C, 20 min at 58°C, and 5 min at 72°C), and 10 min at 72°C for a final extension. One µl of this amplification mixture was reamplified as a template in a PCR reaction with F1-NT/YG-4R and HY-3F/R4-NT primer sets and a 50 µl reaction volume. The PCR conditions were: 2 min at 94°C, followed by 30 cycles (30 sec at 94°C, 1 min at 60°C, 90 sec at 72°C), and 10 min at 72°C for final extension. These amplification products were combined and used to directly transform *G. zeae* protoplasts and generate deletion mutants.

GFP-tagged mutant: The DNA fragment (3.4 kb) carrying the GFP and HYG cassette was amplified from pIGPAPA with primers ICL-F1 and HYG-F1, and transformed into Z3639. Transformant GFPZ3639 constitutively expresses GFP and can be used as a male parent in crosses. GFP was transferred to *Δppg1* and *Δpre2* mutants by crossing either a *Δppg1* or a *Δpre2* mutant with GFPZ3639 to yield GFP*Δppg1* and GFP*Δpre2*.

Transcription assay of pheromone precursors and pheromone receptors: We amplified a GFP sequence (0.97 kb) that has a terminator, but no promoter, from pIGPAPA. The

GFP gene fragment was fused to the HYG cassette (1.4 kb), which was amplified from pIGPAPA with primers HYG-F1-GFP and HYG-R2 (GFP::HYG). Target gene transcription was assayed following modification of the deletion construct (Figure 3.1B). The promoter region of the target gene was amplified with primers F1 and R2-GFP and the 3′ flanking region was amplified with primers F3 and R4. After PCR purification, the promoter and the 3′-flanking region were fused with the GFP::HYG construct in a 25 μl reaction, 1 μl of which was used to produce split markers. Following transformation with the two split markers, we recovered deletion mutants in which GFP expression was controlled by the native promoter of the target gene. Target gene expression could be monitored by screening for GFP expression.

Fungal protoplasting and transformation: Protoplasts of *G. zeae* were transformed with a modified polyethyleneglycol (PEG)-mediated method (Lee *et al.,* 2002). Strain, Z3639 was inoculated in 50 ml of liquid CMC (15 g carboxymethylcellulose, 1 g NH_4NO_3, 1 g KH_2PO_4, 0.5 g $MgSO \cdot 7H_2O$, and 1 g yeast extract for 1 liter) medium and incubated on an orbital shaker (120 rpm) for 5 days at 24°C. The culture was filtered through one layer of Miracloth (Calbiochem, San Diego, CA), and the filtrate was centrifuged for 5 min at 4000 × g at room temperature, 22°C). After discarding the supernatant, conidia were resuspended in 15% glycerol at a final concentration of 10^8/ml, and stored at –70°C. To make fungal protoplasts, 1 ml of the conidial suspension was inoculated in 100 ml YEPD (3 g yeast extract, 10 g peptone, and 20 g glucose for 1 liter) medium, and incubated for 12 to 14 hours at 24°C on an orbital shaker (120 rpm). A protoplasting solution was prepared by dissolving 500 mg Driselase (Sigma, St. Louis, MO) and 100 mg Lysing enzyme (Sigma, St. Louis, MO) in 20 ml of 1.2 M KCl, and stirring for 30 min in ice. The solution was filtered through a 0.2 μm filter (Nalge, Rochester, NY). Mycelia were harvested from the YEPD media by filtration through two layers

of Miracloth. The harvested mycelia were washed with 50 ml of distilled water and washed again with 30 ml of 1.2 M KCl. The mycelia were transferred to 20 ml of protoplasting solution and incubated for 1 hour at 30°C with shaking at 80 rpm. The solution was filtered through two layers of Miracloth, and the filtrate centrifuged at 4000 × g for 4 min at 4°C to pellet the protoplasts. Protoplasts were resuspended in 1×STC (1.2 M sorbitol, 10 mM Tris-HCl (pH 8.0), 50 mM $CaCl_2$) solution with 10^7 protoplasts/ml. DMSO was added for storage at -70°C (final DMSO concentration 7%).

For fungal transformation, two split markers from the final round of PCR were combined. Twenty µl from each amplification reaction was added to 200 µl of the protoplast suspension and the mixture incubated for 20 min on ice. One ml of PTC solution (40% PEG 4000 in 1×STC) was added to the mixture and mixed well and the incubation continued for 20 min. Five ml of TB3 (3 g yeast extract, 3 g casamino acids, and 200g sucrose in 1 liter) media was added to the final mixture which was incubated with shaking (80 rpm) for 15 hrs at room temperature to allow protoplast walls to regenerate. Hyphae were pelleted following centrifugation for 5 min at 4000 × g, and the supernatant discarded. One ml of STC was added to the harvested hyphae and 9 ml of TB3 media containing 0.7% agarose and 100 ng/ml of hygromycin or geneticin were added and plated on a petri dish. After 15 hours, 10 ml of TB3 + 0.7% agarose and 200 ng/ml of drug was overlaid on the plate. After 2 to 4 days, survivors were transferred to Complete medium (Leslie and Summerell, 2006) + 200 ng/ml of drug.

Screening of mutants: Transformants were identified based on growth on Complete medium (CM) + 200 ng/ml of hygromycin. Growing colonies were subcultured on CM + hyg and mutants growing from this subculture were transferred to carrot agar (Leslie and Summerell, 2006). After three days of growth, aerial mycelia were collected with toothpicks and transferred

to a 1.5 ml microcentrifuge tube containing 400 µl CTAB buffer (Leslie and Summerell, 2006) and incubated for 10 min at 60°C. Then 400 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and the tube was shaken at 200 rpm for 1 hour. The tube was centrifuged [5 min, 15,300 × *g*, room temperature] and the supernatant decanted to a clean microcentrifuge tube. DNA was precipitated with 400 µl of ethanol and redissolved in 20 µl of water, 1 µl of which was used for a PCR-based screening reaction. PCR amplification with the F1-/R4 primer pair should amplify fragments of different sizes in the wild type and the mutant strains, i.e., the markers were codominant (Figure 3.2A). When codominant markers were not available, dominant/recessive markers were used from PCR reactions with the F1/YG-4R and F5/R6 primers (Figure 3.2B). Single homologous replacement events were confirmed by Southern hybridizations to genomic DNA with the 5′ flanking region of the target gene as the probe.

Complementation of *ppg1*: Three constructs were made to complement *ppg1*. In the first, the entire *ppg1* gene, including the native promoter and terminator was amplified from *G. zeae* strain Z-3639 with primers PPG1-F1 and PPG1-R-TRPCP, and the geneticin resistant gene cassette (GNT) was amplified from pII99 (Namike *et al*., 2001) with primers GNT-F1-PPG1 and GNT-R4. These two amplicons were fused (whole *ppg1* cassette::GNT) after purification as described above in "targeted gene deletion". In the second, the *ppg1* gene was amplified by PCR from Z3639 with PPG1-F1-ICL and PPG1-R-trpCP as the primers, with the resulting DNA fragment containing the entire *ppg1* sequence and the 3′ flanking region including the terminator. A strong constitutive promoter, ICL from *N. crassa*, was amplified from pIGPAPA (Horwitz *et al.,* 1999) by PCR with ICL-F1 and ICL-R2-PPG1. The ICL promoter fragment, the *ppg1* genomic fragment and the GNT cassette were fused (ICL promoter::*ppg1*::GNT). In the third, the construct is similar to the second construct except that the *ppg1* gene fragment, amplified

with primers PPG1M2-F1-ICL and PPG1-R-TrpCP, begins at the second methionine codon of *ppg1* and does not include the signal peptide. All three constructs were transformed into *Δppg1*.

Crossing and fertility tests: All fungal strains were incubated at 24°C with a 12 hr:12 hr day:night cycle (Leslie and Summerell, 2006). For tests of self-fertility, strains were inoculated on carrot agar (Leslie and Summerell, 2006) and the mycelia mock-fertilized with 500 µl of aqueous 2.5% Tween 60 per plate to induce sexual development. Perithecial development was scored after incubation for at least 10 days. Female fertility was tested by inoculating the strains on carrot agar as described below and estimating the % of fertile perithecia formed 10 days after mock-fertilization. Male fertility was tested by using a suspension of conidia/hyphal fragments from cultures grown on complete media to fertilize a mating type knockout strain as described below and comparing the relative fertility of the mutant and its wild type parent.

Crosses were made as previously described (Bowden and Leslie, 1999). Five hundred µl of a conidial suspension (1×10^5 conidia/ml in aqueous 2.5% Tween 60) served as the male parent and a 3-5 day old culture on carrot agar served as the female parent. For self-fertilization, 500 µl of an aqueous 2.5% Tween 60 solution was used as the spermatizing agent. Perithecia were scored 10 days after induction/fertilization. Double mutants were obtained from crosses between appropriate single mutants. Neither *Δmat1-1* nor *Δmat2* transformants were fertile as homothallics (Lee *et al.*, 2003), so when these strains served as the female parent all of the progeny resulted from heterozygous crosses. Fertility was quantified by inverting a cross plate ten days after fertilization and collecting the ejected ascospores in one ml of sterile water on the inner lid of a Petri dish. Ascospores were counted with a haemacytometer to determine the total number of progeny produced and the number of progeny expressing GFP.

The outcrossing ratio was also quantified. Z3639, *Δppg1*, and *Δpre2* were used as the female parent, and GFP-tagged mutants of these strains were used as the male parent. In these crosses a 1:1 ratio of GFP:non-GFP progeny was not expected since the female strains are capable of self-fertilization. The % of GFP ascospores should be less than 50 % since the GFP gene can be introduced only through the male parent and not through the female parent. The higher the % of GFP progeny in these crosses the greater the % of outcrossing that is occurring.

Light microscopy was performed with an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) and the samples photographed with an AxioCam HRc. GFP was observed in the same system with 480±10 nm for excitation and 510±10 nm for emission.

Outcrossing also was performed in the presence of synthetic *ppg1* peptides from *G. zeae* and *N. crassa*. Peptide sequences were "WCWWKGQPCW" and "QWCRIHGQSCW" for *G. zeae* and *N. crassa*, respectively. Peptides were synthesized by Celtec Peptide Co. (Nashville, TN) and dissolved in 2 % dimethylsulfoxide (DMSO). One hundred µl of a solution containing the synthetic peptide (0, 5, 25, 125, 250, and 500 nmol) in 2% DMSO was spread on a carrot agar plate before inoculation. After the peptide solution was dried, the female parent was inoculated and incubated as described above. Three days later the cultures were fertilized with 500 µl of 2.5 % Tween 60 solution containing 2×10^5 conidia/ml of GFPZ3639 and the proportion of GFP progeny determined 10 days later as described above.

DNA manipulation and Southern hybridization: DNA was extracted with a cetyltrimethyl ammonium bromide (CTAB) procedure (Leslie and Summerell, 2006). Standard procedures were used for restriction endonuclease digestions, agarose gel electrophoresis and Southern hybridizations (Sambrook *et al.,* 1989).

RNA isolation and RT-PCR: Total RNA was isolated from vegetative mycelia or ascospores of wild type and mutant strains by using TRIZOL reagent (Invitrogen, Carlsbad, CA). Fungal strains were inoculated on carrot agar (Leslie and Summerell, 2006). Mycelia were harvested 3 days after inoculation, and 3 to 6 days after induction. Ascospores were harvested 10 days after induction. Samples were ground to a powder under liquid nitrogen in a mortar with a pestle, placed into a 1.5 ml microfuge tube, and 1 ml of TRIZOL reagent was added to each ~ 50 mg of sample. The homogenized samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, then 0.2 ml of chloroform was added and the mixture shaken vigorously by hand for 15 sec. The samples were incubated for 2 min at room temperature and then centrifuged at 12,000 × g for 14 min at 4°C. The aqueous phase was transferred to a fresh tube, and 0.5 ml of isopropyl alcohol was added and incubated at room temperature for 10 min to precipitate nucleic acids. Total RNA was pelleted by centrifugation at 12,000 × g for 10 min at 4°C. After removing the supernatant, the RNA pellets were rinsed with 75% ethanol, dried at 55 °C for 10 min, and resuspended in 100 µl of DEPC-treated water. RNA concentration and purity were checked using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). For RT-PCR, first-strand cDNA was synthesized with 3′-full RACE Core Set (Takara Bio Inc, Japan) as described in manufacturer's instructions.

Results

Structure of *ppg1*, *ppg2*, *pre1*, and *pre2*: Only one putative *ppg1* (gene annotation name in the Broad Institute; FG05061.1) sequence was identified. This protein sequence had significant similarity (29, 32, and 33%, respectively) to *ppg1* of *S. macrospora*, *ccg4* of *N. crassa*, and *mf2-1* of *M. grisea*. Upstream of FG05061.1 there are MAT binding motifs (CTTTG) at positions –434 and –483. The putative *ppg1* gene contains four repeats of one

decapeptide (WCTWKGQPCW) and five repeats of a second decapeptide (WCWWKGQPCW) that differs from the first decapeptide by a single amino acid (Figure 3.3). All decapeptides except for the first two are bordered by a putative Kex2 protease site (KR).

ppg2 could not be unambiguously identified in a BlastP search with *ppg2* of *S. macrospora*, *mfa-1* of *N. crassa* or *mf1-1* of *M. grisea*, because the sequences are small and have relatively low levels of sequence similarity. We compared synteny of the *N. crassa* and *M. grisea* sequences flanking the *mfa-1* and *mf1-1* genes. Both genes are between cyanate lyase and *ebp2* homologs, and all three *ppg2* homologues are transcribed in the same direction and in the same open reading frame (Figure 3.4). Ten kb of sequence flanking *mfa-1* of *N. crassa* and *mf1-1* of *M. grisea* was blasted against the genomic sequence of *G. zeae*. Contig 310 contains a homolog of cyanate lyase (FG07458.1) and *ebp2* (FG07457.1), but no gene had previously been identified in the region between the two genes. The putative *ppg2* gene encodes a peptide of 21 amino acids with a prenylation signal sequence (CAAX) at its C-terminus (Figure 3.5). This signal sequence also is found in the precursors of several other fungal pheromones (Andregg *et al.*, 1988; Davey, 1992; Olensnichy *et al.,* 1999; Pöggeller, 2000; Shen *et al.*, 1999; Spellig *et al.*, 1994). Upstream of the putative *ppg2* gene there is a putative TATA box at position –142 and a putative MAT gene binding site (CAAAG) at position –633 (Figure 3.5).

FG07270.1 is a putative *pre1* gene from *F. graminearum* with high sequence identity (24, 32 % identity) to the *preA* gene in *E. nidulans* (GenBank DAA01795) and the *pre1* gene of *N. crassa* (GenBank CAC86413.1). The putative *F. graminearum pre1* gene has a CAAAG motif at –440 and encodes a protein with seven transmembrane domains (Figure 3.6A).

FG02655.1, a putative α-pheromone receptor (*pre2*), has high sequence similarity (25, 37%) with *preB* from *E. nidulans* (GenBank DAA01796) and *pre2* gene from *N. crassa*

(GenBank CAC86431.1). The *F. graminearum pre2* sequence has a CAAAG motif at positions –506 and –526 and seven transmembrane domains (Figure 3.6B).

Phenotype of deletion mutants: Deletions of *ppg1* (*Δppg1*, 11 mutants), in which the entire *ppg1* gene was deleted by replacing it with HYG, produced fewer perithecia than did the wild type parent. Approximately 70% of the perithecia produced by the *Δppg1* mutants failed to mature (Figure 3.7). These immature perithecia were darkly pigmented, but smaller than mature perithecia and contained neither asci nor ascospores. These immature perithecia never developed further, even if the cultures were incubated for 3 additional weeks or if wild type conidia were placed around them. *Δpre2* (13 mutants) mutants had a phenotype similar to that of *Δppg1,* but the number of immature perithecia, ~ 60%, was somewhat less than in *Δppg1*. *Δppg2* (19 mutants) and *Δpre1* (11 mutant) mutants had no detectable sexual function or morphological changes.

The *Δppg1*/*Δppg2* double mutant was generated by crossing *Δppg1*/*Δmat2* with *Δppg2*. All single (*Δppg1* or *Δppg2*) and double mutants (*Δppg1*/*Δppg2*) could serve as either the male or the female parent in a cross (Table 3.3).

Complementation test: *Δppg1* deletions were made with a construct that contained *hyg* as the selectable marker. To test for complementation, the entire *ppg1* gene including the 5′ and 3′ flanking regions, was fused with a geneticin resistant cassette (GNT) and geneticin-resistant transformants selected following transformation into a *Δppg1* mutant. All seven of the geneticin resistant transformants had a wild type phenotype. If the reintroduced *ppg1* sequence was controlled by the constitutive ICL promoter from *N. crassa* (ICL promoter::*ppg1*::GNT), then partial complementation (70–80% mature perithecia produced) occurred in each of the five

transformants. Thus the timing or amount of *ppg1* expression is important for detecting its function.

Eight transformants carried the *ppg1* gene but lacked the initial signal peptide under the control of the ICL promoter. Strains carrying this construct did not complement the *ppg1* deletion and had the same phenotype as the *Δppg1* mutants. Thus the signal peptide of *ppg1* is essential for the *ppg1* gene product to function properly.

Gene expression: In four *Δppg1* GFP mutants, in which GFP expression was controlled by the native *ppg1* promoter, GFP was expressed strongly in germinating conidia and mature ascospores (Figure 3. 8). Mycelia and conidia from colonies > 10 days old on carrot media had a weak GFP signal. Young mycelia, perithecia, ungerminated conidia, and ascospores inside asci had no detectable GFP signal. We never observed GFP expression in the *Δppg2* GFP mutants (9 mutants). GFP was weakly expressed in mature ascospores of *Δpre1* GFP mutants (4 mutants) and was more strongly expressed in *Δpre2* GFP mutants (7 mutants) by all mycelial ages or tissues from carrot agar except young mycelia prior to induction (Table 3.4).

Male fertility of *Δppg1* and *Δpre2*: In other ascomycete fungi, the *ppg1* pheromone attracts hyphae carrying *MAT1-2* (Coppin *et al.*, 2005). Z3639, *Δppg1*, and *Δpre2* strains were tagged with GFP. Different combinations for outcrossing among them were made and the amount of outcrossing determined. Combinations of a female *Δppg1* strains with a male wild type strain or *Δpre2* mutant resulted in high levels of outcrossing (Table 3.5), suggesting that the *ppg1*/*pre2* interaction increases outcrossing in *G. zeae*.

Outcrossing fertility in the presence of synthetic peptides: Outcrossing fertility was negatively correlated with the concentration of α-factor-like synthetic peptides by *G. zeae* and *N.*

crassa (Figure 3.9). The ability of a *Δpre2* mutant to outcross also was reduced by high levels of these peptides. However, outcrossing was not completely blocked by the synthetic peptides.

Discussion

Putative pheromone precursor genes (*ppg1* and *ppg2*) and pheromone receptor genes (*pre1* and *pre2*) found in heterothallic ascomycetes also are present in the homothallic ascomycete *G. zeae*. *ppg1* was strongly expressed in germinating conidia and ascospores discharged from perithecia, but we never detected the expression of *ppg2*. This expression pattern is quite different from that found in heterothallic ascomycetes, in which pheromone precursor gene expression is mating-type allele specific (Zhang *et al.,* 1998; Shen *et al.,* 1999; Bobrowicz *et al,*. 2002), the conidia constitutively express the pheromone precursor genes (Bistis, 1981; Bobrowicz *et al.*, 2002), and the expression of these genes is influenced by nitrogen starvation (Bobrowicz *et al.,* 2002; Shen *et al.,* 1999).

The role of carrot agar in the expression of *ppg1* also is unusual. Mycelia and germinating conidia from induced cultures, i.e. cultures growing on carrot agar that have been fertilized with a spore suspension or mock-fertilized with a 2.5% aqueous Tween 60 solution, express *ppg1*. However, conidia germinating from cultures grown on water agar, complete media (solid or liquid), minimal media (solid or liquid), minimal media containing 10% of the normal nitrogen, and CMC liquid media do not express *ppg1*. A few conidia from colonies more than 10 days old growing on *Neurospora* synthetic crossing media express *ppg1* when they germinate. However, neither aerial mycelia, pigmentation, nor perithecia were produced on this medium. If ungerminated conidia from induced cultures are transferred to fresh media (water agar, minimal media, complete media, or carrot media) for germination, then *ppg1* expression

was not detected (data not shown). Thus the induced colony appears to be reacting to a very specific compound(s) present in the carrot agar that induces the expression of *ppg1*.

G. zeae has both MAT idiomorphs on the same chromosome, and both idiomorphs must function for sexual reproduction to occur (Yun *et al.*, 2000; Lee *et al.*, 2003). We expected both pheromone precursor genes to be expressed as they are in the homothallic ascomycete *S. macrospora* (Pöggeler, 2000). We found *ppg1* to be highly expressed, but we never observed *ppg2* expression. We do not know if the putative *ppg2* sequence we identified has pheromone function, although the genomic sequences of *N. crassa*, *M. grisea* which are syntenous with *G. zeae* in this region, contain *ppg2* homologs that are expressed (Bobrowicz *et al.*, 2002; Shen *et al.*, 1999), and *pre1*, which may interact with *ppg2*, is at most weakly expressed.

ppg1 also could act as a regulator for conidial germination. In *N. crassa*, conidial germination is inhibited by the *ppg1* homolog. In *G. zeae*, conidial germination also is inhibited by the *ppg1* pheromone peptide (Chapter 4). *ppg1* expression in ascospores suggests that these propagules could compete with conidia for protoperithecia to fertilize. Such a mechanism could help maintain genetic diversity since ascospores could be genetically diverse while conidia will certainly be clones of the parental colony from which they were derived.

ppg1 and *pre2* have a role in the fertility of both self-crosses and outcrosses. On an outcrossing plate, female conidia produced *in situ* usually outnumber the male conidia added as the fertilizing parent. The male conidia are added in such a manner that all of the conidia are induced when the male conidia are added to the plate. If a wild-type female is fertilized by a wild-type male, then the amount of outcrossing should be similar to the ratio of female:male conidia. Under these conditions more female conidia should be present than male conidia, leading to an excess of apparently "selfed" perithecia relative to the number of heterozygous

outcrossed perithecia. If the female strain is *Δppg1* and the male strain is wild type, then the conidia from the female parent will not produce the *ppg1* pheromone and the relative number of outcrossed perithecia should increase (Table 3.5). If a female *Δppg1* strain and a male *Δpre2* strain are the parents, then the outcrossing ratio is similar to that between a *Δppg1* female and a wild type male because the *Δpre2* male parent still has a functional *ppg1*. When a *Δpre2* strain is used as the female parent, the presence/absence of *ppg1* is irrelevant, as the female strain lacks the pheromone receptor (*pre2*) required to respond to *ppg1* and the amount of outcrossing is unaffected.

The presence of exogenous, synthetic α-factor-like peptides similar to those produced by *G. zeae* and *N. crassa* also affected the outcrossing fertility of *G. zeae* (Figure 3.9). We expected that the amount of outcrossing would decrease in the presence of excess amounts of these synthetic peptides since they should saturate the *pre2* receptor. Outcrossing was not completely blocked by a high concentration of these peptides, even though it was decreased. The fertility of the *Δpre2* strains, which lack the target receptor, was reduced in the presence of excess peptides, suggesting that *G. zeae* may have one (or more) additional peptide receptors in addition to the *pre2* receptor. The role of this receptor(s) in sexual reproduction is unknown. The synthetic peptides also inhibit spore germination by all *G. zeae* strains tested, including *Δpre2* (Chapter 4). Thus, the second receptor could be involved in the spore germination inhibition process and the decrease in outcrossing resulting from treatment with these peptides could be a side effect of the inhibition of spore germination.

Perithecial development in *G. zeae* has been well described (Trail and Common, 2000), but trichogynes have yet to be described for *G. zeae*. The immature perithecia we saw in *Δppg1* and *Δpre2* cultures on carrot agar are not the equivalent of the protoperithecia of *N. crassa*

described by Bistis (1981, 1983) in that they neither produce visible trichogynes nor do they interact with conidia placed near them. The protoperithecial equivalents in *G. zeae*, if they occur at all, occur at an earlier time and may be difficult to distinguish given the relatively dense mycelial growth on carrot agar prior to fertilization. At this time we do not know how the conidia that serve as the male parent are recognized and their nuclei transported to a protoperithecium that later develops into a cross-fertile perithecium. *ppg1* and *pre2* are certainly involved in this process, given the reduction in self-fertility that results whenever one or both of these genes is inactivated. That the amount of self-fertilization is not zero, however, suggests that *G. zeae* has an alternate fertilization strategy that can be used only for self-fertilization.

In conclusion, one of the pheromone/receptor pairs (*ppg1*/*pre2*) found in many Ascomycetes has a role in, but is not essential for, selfing or outcrossing in *G. zeae*, whereas the other pheromone/receptor pair (*ppg2*/*pre1*) no longer has a detectable function in sexual reproduction. We did not discern the exact functional mechanism of the *ppg1*/*pre2* pair or the stage of the process that they influence, e.g., initial interaction, cellular fusion, nuclear recognition or nuclear migration, but the pheromone pathway (*ppg1*/*pre2*) of *G. zeae* should be an excellent system for these studies since *Δppg1* or *Δpre2* still retains both male and female functions.

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Table 3.1 *G. zeae* strains used in this study

Strain[a]	Original strain		Genotype
	Paternal	Maternal	
Z3639	Wild type		Wild type
Δmat1-1	Z3639		*Δmat1-1-1*
Δmat2	Z3639		*Δmat1-2*
Δppg1	Z3639		*Δppg1*
Δppg2	Z3639		*Δppg2*
Δpre1	Z3639		*Δpre1*
Δpre2	Z3639		*Δpre2*
GFPZ3639	Z3639		Z3639 tagged with GFP
GFP*Δppg1*	GFPZ3639	*Δppg1*	*Δppg1* tagged with GFP
GFP*Δpre2*	GFPZ3639	*Δpre2*	*Δpre2* tagged with GFP
*Δppg1*GFP	Z3639		*Δppg1* for transcription assay
*Δppg2*GFP	Z3639		*Δppg2* for transcription assay
*Δpre1*GFP	Z3639		*Δpre1* for transcription assay
*Δpre2*GFP	Z3639		*Δpre2* for transcription assay
Δppg1/*Δmat1-1*	*Δppg1*	*Δmat1-1*	*Δppg1* and *Δmat1-1-1*
Δppg1/*Δmat2*	*Δppg1*	*Δmat2*	*Δppg1* and *Δmat1-2*
Δppg2/*Δmat1-1*	*Δppg2*	*Δmat1-1*	*Δppg2* and *Δmat1-1-1*
Δppg2/*Δmat2*	*Δppg2*	*Δmat2*	*Δppg2* and *Δmat1-2*
Δppg1/*Δppg2*	*Δppg2*	*Δppg1*/*Δmat2*	*Δppg1* and *Δppg2*
CNP*Δppg1*	*Δppg1*		Complementation of *ppg1* with native promoter
CSP*Δppg1*	*Δppg1*		Complementation of *ppg1* under Icl promoter
CSP2S*Δppg1*	*Δppg1*		Complementation of *ppg1* under Icl promoter in which *ppg1* did not contain signal peptide

[a] All mutant strains except for *Δmat1-2* mutant were generated for this study, and *Δmat1-2* was kindly provided by Prof. Yin-Won Lee in Seoul National University, Seoul, Korea.

Table 3.2 Oligonucleotides used in this study

Name	Sequence (5'-3')
PPG1-F1	CGC GTC TGA CAA GTA AAA GGA GAA AC
PPG1-F1-NT	GCG TCT GAC AAG TAA AAG GCG AAA CCA AAT GGC AA
PPG1-R2	AAA AAG TGC TCC TTC AAT ATC ATC TTC TGA GGC GGC TAG CGT CAA AAT GGA
PPG1-F3	CTT GTT TAG AGG TAA TCC TTC TTT CTA GAG TAA GTT TGG TTA TCG ACG CAG AG
PPG1-R4	CTA GCG CAC AAG GCA TCA ACT
PPG1-R4-NT	CTG CCC CAC CAT CTC AGA CGC
PPG1-R2-GFP	CCT CGC CCT TGC TCA CCA TGT T GG GCG CCG TAC TTG TCG
PPG1-R-TRPCP	GCT CCT TCA ATA TCA TCT TCT G GG ATA TGC TGC TTG GCG TCA AC
PPG1-F1-ICL	TTC ATA CCA CAC CTG CCC ACC GCG CCC AAC ATG AAG TAC TCC
PPG1M2-F1-ICL	TTC ATA CCA CAC CTG CCC ACC ATG CCC TGG TGC ACC TGG AAA
PPG2-F1	GGCCGCCAAAGACCTAAGC
PPG2-F1-NT	GCGGTGGCAGCATTGTTCTACG
PPG2-R2	TTG ACC TCC ACT AGC TCC AGC CAA GCC GGC CAG AAC GAT ACG TC
PPG2-F3	GAA TAG AGT AGA TGC CGA CCG CGG GTT CCG CCA CGA GGA CGC CA
PPG2-R4	GCG AGG AGG GCG GTT GTG TTG TTA
PPG2-R4-NT	GGG GCT CTT CAT CGT CCT CAT CAT
PPG2-R2-GFP	CGC CCT TGC TCA C CA TTT TGA AAG TTG GGG TTG AAA GAC TTA GA
PRE1-F1	GCT GAG ACG CGA TAG GGT AGG AA
PRE1-F1-NT	TCA ACC TCA CCA CGT CCC TCA ACA
PRE1-R2	TTG ACC TCC ACT AGC TCC AGC CAA GCC GCG GGA AGC GCA AGG AC
PRE1-F3	GAA TAG AGT AGA TGC CGA CCG CGG GTT CGG CAT CAT CTG CGA GC
PRE1-R4	GAC GAA CGT ATG CGA AAT GGA GAC
PRE1-R4-NT	AAG GGC TTG GTT ATG GCG GTT GG
PRE1-F5	CCT GGA CCG GGA ACA ACT ATC ACT
PRE1-R6	AGG CCA TGC GAC CCA ACT G
PRE1-R2-GFP	CGC CCT TGC TCA CC A TGT TGG GAC GTC GAC CGT GAT GTG GAA G
PRE2-F1	GCG CAG CAG GCA GCA GAA
PRE2-F1-NT	GAA TGG GCC TGG CTG CGT GAT
PRE2-R2	TTG ACC TCC ACT AGC TCC AGC CAA GCC GGC AAG AAG ACA CGG GA
PRE2-F3	GAA TAG AGT AGA TGC CGA CCG CGG GTT CCG CCT CGA TAC CCC AA
PRE2-R4	TCG CCA CAA TTC GGT TCC TGA T
PRE2-R4-NT	GGA ACC CCG GTC GCC TCA CA
PRE2-F5	TGT CTT ATC ATG CTG GTC GTG CTC
PRE2-R6	GGG AGA ATC ACA GCG ACA GAG GTA
PRE2-R2-GFP	CGC CCT TGC TCA CCA TGT TGG GGT GGT ATC TGC TTT TCG ACT GG
MAT11-F1	CTC CAC TTG CGG CAT CGT CTA C
MAT11-F1-NT	GCC CTG ATG ATG CTG TAA GTG TTA

MAT11-R2	TTG ACC TCC ACT AGC TCC AGC CAA GCC GGA GGG AAA GGG GTG TG
MAT11-F3	GAA TAG AGT AGA TGC CGA CCG CGG GTT GCA CAT GTC GGG CAC GG
MAT11-R4	CTC CCA ACG CTT ACA TCC TCT ACC
MAT11-R4-NT	CCC GCC GCC CAG CCT ACT C
ICL-F1	GGG CCC CAC ACG GAC TCA AAC
ICL-F1-NT	CCC CAC ACG GAC TCA AAC TGA TGT TCG AGT C
ICL-R2-PPG1	GGA GTA CTT CAT GTT GGG CGC GGT GGG CAG GTG TGG TAT GAA A
ICL-R2-PPG1M2	CCA GGT GCA CCA GGG CAT GGT GGG CAG GTG TGG TAT GAA A
GFP-F1	GGG GCC CCA CAC GGA CTC
GFP-F1-NT	CCA GAG GTC CGA TCG CCA ATG A
GFP-R2	TTG ACC TCC ACT AGC TCC AGC CAA GCC AGA TGA CAC CGC GCG CG
GFP-F3	CCA TGG TGA GCA AGG GCG AGG AG
GFP-F3-PPG1	ACG ACA AGT ACG GCG CCC AAC ATG GTG AGC AAG GGC GAG G
GFP-F3-PPG2	CTT TCA ACC CCA ACT TTC AAA ATG GTG AGC AAG GGC GAG G
GFP-F3-PRE1	TCC ACA TCA CGG TCG ACG TC A TGG TGA GCA AGG GCG AGG
GFP-F3-PRE2	CGA AAA GCA GAT ACC ACC ATG GTG AGC AAG GGC GAG G
GFP-R4	AGA TGA CAC CGC GCG CGA TAA TTT A
HYG-F1	GGC TTG GCT GGA GCT AGT GGA GG
HYG-R2	AAC CCG CGG TCG GCA TCT ACT CTA
HY-F3	GAT GTA GGA GGG CGT GGA TAT GT
YG-R4	GAA CCC GCT CGT CTG GCT AAG
HYG-F1-GFP	TAT CGC GCG CGG TGT CAT CT G GCT TGG CTG GAT CTA GTG GAG G
GNT-F1	CAG AAG ATG ATA TTG AAG GAG C
GNT-R4	CTA GAA AGA AGG ATT ACC TCT
GNT-F1-PPG1	CAA CGT TGA CGC CAA GCA GCA TAT CC C AGA AGA TGA TAT TGA AAG
GNT-R4-NT	CCT GTG CAT TCT GGG TAA ACG AC
GNT-R4A-NT	GTA CCT GTG CAT TCT GGG TAA ACG ACT CAT AGG AG

Table 3.3 Male and female fertility of mutant strains

Male	Female					
	Δmat1-1	*Δmat1-2*	*Δppg1/ Δmat1-1*	*Δppg1/ Δmat1-2*	*Δppg2/ Δmat1-1*	*Δppg2/ Δmat1-2*
Z3639	+[a]	+	+	+	+	+
Z3639-G2	+	+	+	+	+	+
Δppg1	+	+	+	+	+	+
Δppg2	+	+	+	+	+	+
Δppg1/Δmat1-1	–[b]	+	–	+	–	+
Δppg1/Δmat1-2	+	–	+	–	+	–
Δppg2/Δmat1-1	–	+	–	+	–	+
Δppg2/Δmat1-2	+	–	+	–	+	–
Δppg1/Δppg2	+	+	+	+	+	+
Mock-fertilization[c]	–	–	–	–	–	–

[a] Plus (+) ascospores produced.

[b] Minus (–) no ascospores produced.

[c] Female was self-fertilized without male conidia.

Table 3.4 Expression profiles of pheromone precursors (*ppg1* and *ppg2) and receptors (*pre1* and *pre2*)**

	ppg1	*pre1*	*pre2*
3 DAI[a] (before induction[b])			
Mycelia	–[c]	–	–
Conidia	–	–	+
6 DAI (3 days after induction)			
Mycelia	–	–	++
Ungerminated conidia	–	–	++
Germinating conidia	++++[d]	–	++
Young perithecia	–	–	+++
13 DAI (10 days after induction)			
Mycelia	–	–	++
Ungerminated conidia	–	–	++
Germinating conidia	++++	–	++
Mature perithecia	–	–	++
Ascospores in perithecia	–	–	+
Ascospores discharged from perithecia	+++	+	+
Old culture without induction (15 DAI)			
Mycelia	+	–	++
Ungerminated conidia	+	–	++
Germinating conidia	++++	–	++
Mature perithecia	–	–	++
Ascospores in perithecia	–	–	+
Ascospores discharged from perithecia	+++	+	+

* Expression of *ppg2* was not detected in any cells.

[a] Days after inoculation.

[b] Note that knockdown mycelia to induce sexual development with 500 µl of 2.5% Tween 60 solution.

[c] Signal not detected.

[d] Relative strength of GFP signal.

Table 3.5 Outcrossing fertility among Z3639, *Δppg1*, and *Δpre2* strains

Female	Male[a]	Percentage of GFP-ascospores[b] (standard deviation)	
		Conidia[c]	Ascospores[d]
Z3639	GFPZ3639	0.13 (0.07)	0.98 (0.71)
Z3939	GFP*Δppg1*	0.14 (0.12)	0.14 (0.04)
Z3939	GFP*Δpre2*	0.53 (0.21)	2.07 (1.24)
Δppg1	GFPZ3639	17.66 (5.82)	30.81 (4.58)
Δppg1	GFP*Δppg1*	0.64 (0.32)	0.26 (0.05)
Δppg1	GFP*Δpre2*	26.56 (5.85)	43.35 (5.32)
Δpre2	GFPZ3639	0.20 (0.18)	0.18 (0.11)
Δpre2	GFP*Δppg1*	1.06 (0.40)	2.46 (1.18)
Δpre2	GFP*Δpre2*	0.44 (0.26)	0.76 (0.23)

[a] All male parent were tagged with GFP.

[b] Percentage of GFP-expressing progeny.

[c and d] Conidia and ascospores of male parent were used for male function, respectively.

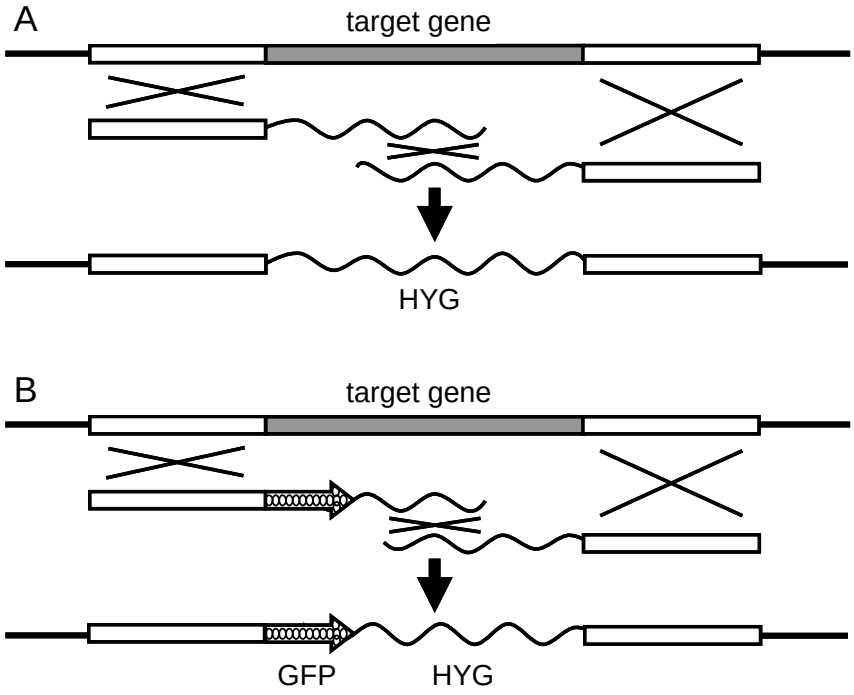

Figure 3.1 Diagram for targeted gene deletion. (A) Targeted gene deletion with hygromycin phosphotransferase cassette (HYG). The 5′ and 3′ flanking regions of a target gene were fused with HYG to make split markers. When the two split markers were introduced into *G. zeae* strain, triple crossing-over may occur between fungal chromosome and split markers to trigger targeted gene deletion. (B) Targeted gene deletion for transcription assay. GFP without its own promoter was fused with 5′ flanking of a target gene. GFP expression in deletion mutants should be controlled by a native promoter of the target gene.

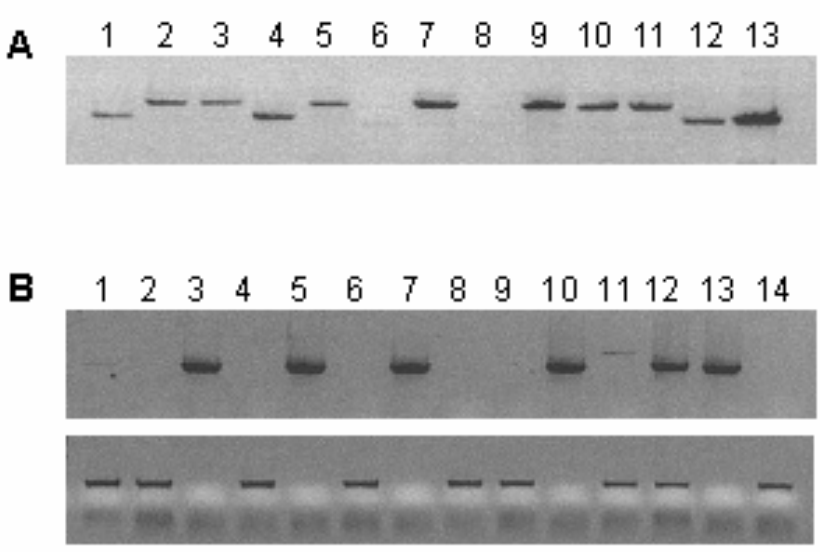

Figure 3.2 Screening of transformants. (A) Codominant markers amplified by PPG1-F1/PPG1-R4 to screen *ppg1*-deletion. Lane 1 to 12 are mutants and lane 13 is a wild type strain, Z3639. Lane 2, 3, 5, 7, 9, 10, and 11 are desirable mutants. (B) Recessive and dominant markers amplified with PRE2-F1/YG-4R and PRE2-F5/R6, respectively, to screen *pre2*-deletion. Lane 1 to 13 are mutants and lane 14 is a wild type strain, Z3639. Lane 3, 5, 7, 10, and 13 are desirable deletion mutants. Lane 12 is ectopic.

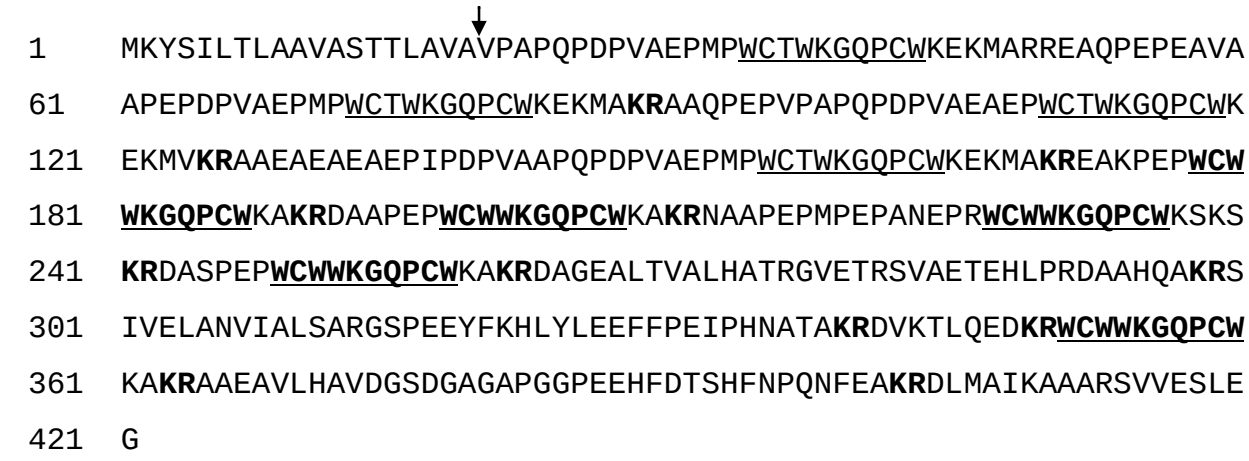

Figure 3.3 The deduced protein of *ppg1* (FG05061.1) from *G. zeae.* This polypeptide contains two types of decapeptides. Four repeats of one type (WCTWKGQPCW) are underlined and five repeats of a second type (WCWWKGQPCW) that differs from the first decapeptide by a single amino acid are underlined in boldface type. The putative signal sequence cleavage site is marked by a vertical arrow. KR dipeptides which are potential Kex2 protease-processing sites are in bold type.

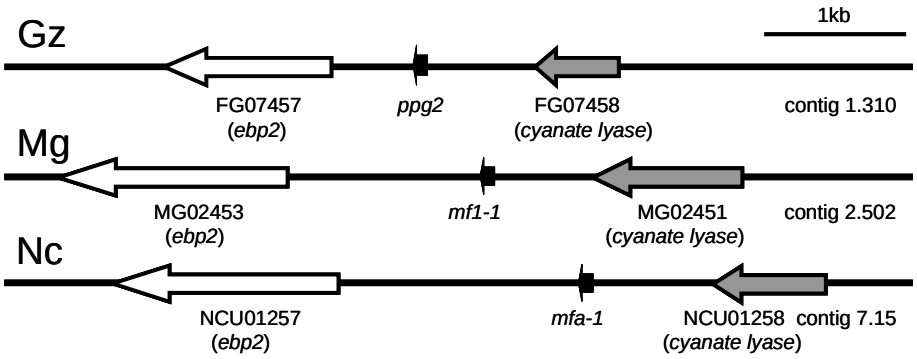

Figure 3.4 Microsynteny near *ppg2* gene among different fungal species. The putative *ppg2* gene of *G. zeae* (Gz) was between FG07457 (*ebp2* homolog) and FG07458 (cyanate lyase homolog) in contig 1.310 of the Fusarium genome database developed by the Broad Institute. In the other fungal species (Mg, M. grisea; Nc, N. crassa), *ppg2* gene was between homologs of *ebp2* and cyanate lyase. Names for the *ppg2* gene are different in these three fungal species.

```
1          ACTCATAACTAACCAACATGTTCAGGCTCCCTTTCAGTCGTTTCTAGGCATTGTTCTCTCAAGAGAATTG
71         GTTGATAGCTCGGAGTTATAACCACTCTGCATGCTGTGTCACTTTCATTAATGACTACTCAAAGCGTATT
141        TACATAACGGAAAATGCGGGTTGGAGCATCGCTAAGTTCACACAAGCCTCACTCAAGAATGACATGTATT
211        TCGAGTAAAGCGGACGGGGCAGGTTGTGGCAATGCGCGTGAAGACAATATGAAGCGAGAGGACAATACTA
281        AGCAATTTACAAACAAATACCAACATTGAGTTCTAGCGCTTCGACGTCCGTGTACCACATGACATGACAA
351        CTCGGCGCATTTCTAAAACCCGAAGGCTTCTAAAGCCTAACTTTCATCTAAAAGAAGGCATGCCAGACAG
421        TCTAGAAGACCTGTGACAAGTTGGTGGCTGCATTAGCGGCGGGTATTCTTTTGTCTGGTAGCAACACCAT
491        CTCGAGAACAATATTGAGCGATTTTGCACGGATCATGGGCTTCACGGCCAACACCAGAACTCAGTGAGTG
561        AGACGTATCGTTCTGGCCAAAATCGAGGGCATCTTGAGTCAGGCAAGTTGATCAGATTCGAGTATAAAGA
631        TGATGGCTTCTTCCTCCCAAATATCATTCTCACCAACAATCACTCACTTCACACACTCACAACACTCTTT
701        TCACCATTCTCAACACAGAACACTCTCCGACTTTTCTAAGTCTTTCAACCCCAACTTTCAAAATGCCTTC
                                                                    M  P  S
771        CACCAAGCCCACCTCTTCCCAGAAGCCCGGCTACCCCCTCAGCTGCACTGTCATGTAAAGCAGCTTTGCT
             T  K  P  T  S  S  Q  K  P  G  Y  P  L  S [C  T  V  M] *
841        TCCCTGATCACTTCTGGACTTTCTTTTCTGAGAGTGACTTGACAACCAACAACGCATGGCAAAGCGTTAG
911        CTCTCAGGCGACAAATTGGAGGAAAATGGAGATGATATGGCAAGGAGAGGATTCATTGGCTTGTTTACGT
981        TTTGTTTTTACCTGGCTCTAAGTGACGGGACACCGCCACGAGGACGCCATTTTACACTGGCTGTCCACTC
```

Figure 3.5 Putative *ppg2* gene of *G. zeae*. The nucleotide sequence is from genome database contig 1.320. The putative MAT gene binding motif (GTTTC or CAAAG), CAAT box, and TATA box are underlined, and the prenylation signal sequence (CAAX) is boxed. The amino acid sequence is indicated below the nucleotide sequence.

A

1 MADSIHLFGRDDLSIIKSPGPGTTITPS**LTANLVCRVLFGIIANFACIVPL**KNLYRNGE**FAAVVFIANIQ**

71 **VSNLDTIINALI**WRDDNTSKWWSGQGFCDVS**PYYTNFLNALFGTCLLAIMRNLA**QQVGLLRANLLSVQEK

141 RRRN**LVQALIMFPLPILQVAWVWPLTM**QRYAVATLVGCSWVAWPAW**PYMAFFVIAPVVVALITSGYAIL**T

211 YIRFREIARTTRTAINSSRSANQRAQRTKRRLY**LMVLAILVPYLPVVITLAVLNIL**GAFPLQPFDYDLIH

281 NRTWPYPWSSVILVPSNGF**TFILLNNCYINILAAIPVVLFFG**MTKDAINSYRLGLLYFGLGHLFPKLQEE

351 YDPDRMIYGSNSGTSHLIDSSVST

B

1 MSKEVFDPFTQNVTFFAPDGKTEISIPVAAIDQVRRMMVNT**TINYATQLGACLIMLVVLLVMVP**KEKFRR

71 **PFMILQITSLVISCCRMLLLSIF**HSSQFLDFYVFWGDDHSR**IPRSAYAPSVAGNTMSLCLVISV**ETMLMS

141 QAWTMVRLWPNVWK**YIIAGVSLIVSIMAISVRLAYTI**IQNNAVLKLEPAFH**MFWLIKWTVIMNVASISWW**

211 **CAIF**NIKLVWHLISNRGILPSYKT**FTPMEVLIMTNGILMIIPVIFA**SLEWAHFVNFESA**SLTLTSVAVIL**

281 **PLGTLAA**QRIASSAPSSANSTGASSGIRYGVSGPSSFTGFKAPSFSTGTTDRPHVSIYARCEAGTSSREH

351 INPQGVELAKLDPETDHHVRVDRAFLQREERIRAPL

Figure 3.6 Putative *pre1* (A, gene annotation no.: FG07270.1) and *pre2* (B, gene annotation no.: FG02655.1) genes of *G. zeae*. The putative transmembrane domains, which were predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), are in boldface type.

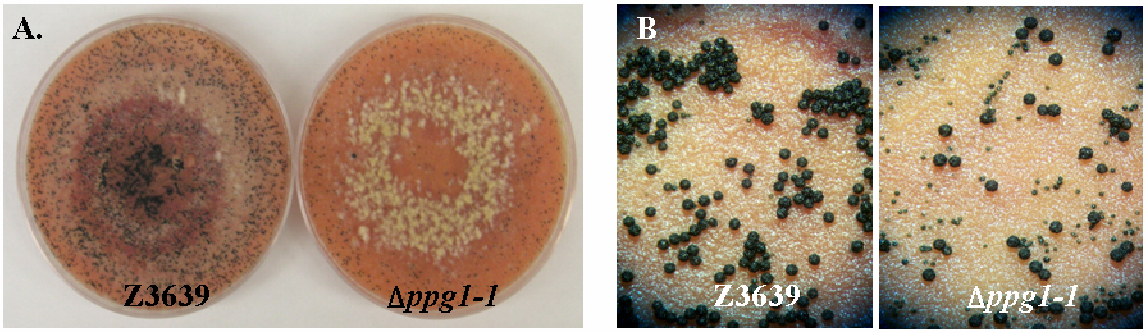

Figure 3.7 Phenotype of *Δppg1* in *G. zeae*. (A) Carrot agar media at 10 days after induction. (B) Perithecia from the same culture as in (A) at 80 × magnification. Z3639, wild type *G. zeae* strain; *Δppg1-1*, *ppg1*-deletion mutant.

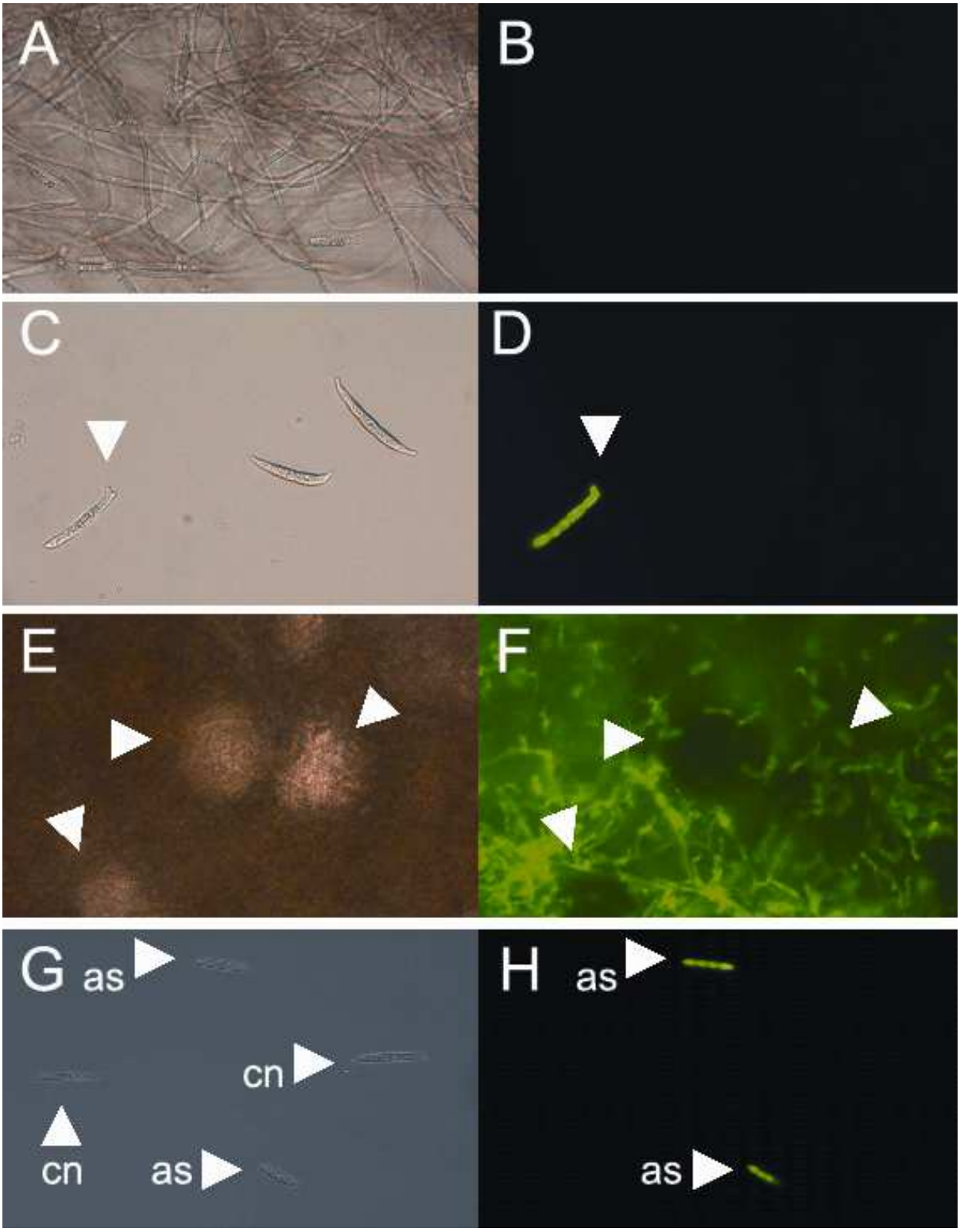

Figure 3.8 Transcription assay of *ppg1*. Expression of GFP was controlled by the native promoter of *ppg1* gene. (A) and (B) Mycelia 1 day after induction. No GFP signal was

detected. (C) and (D) Conidia 3 days after induction. Only a germinating conidium (arrowhead) expressed GFP. (E) and (F) Young perithecia (arrowheads) 3 days after induction. No GFP expression was detected in young perithecia but there were many germinating conidia expressing GFP around them. (G) and (H) Conidia and ascospores 10 days after induction. Ascospores (as) expressed GFP while conidia (cn) did not express GFP. (B), (D), (F), and (H) were observed by a fluorescent microscopy.

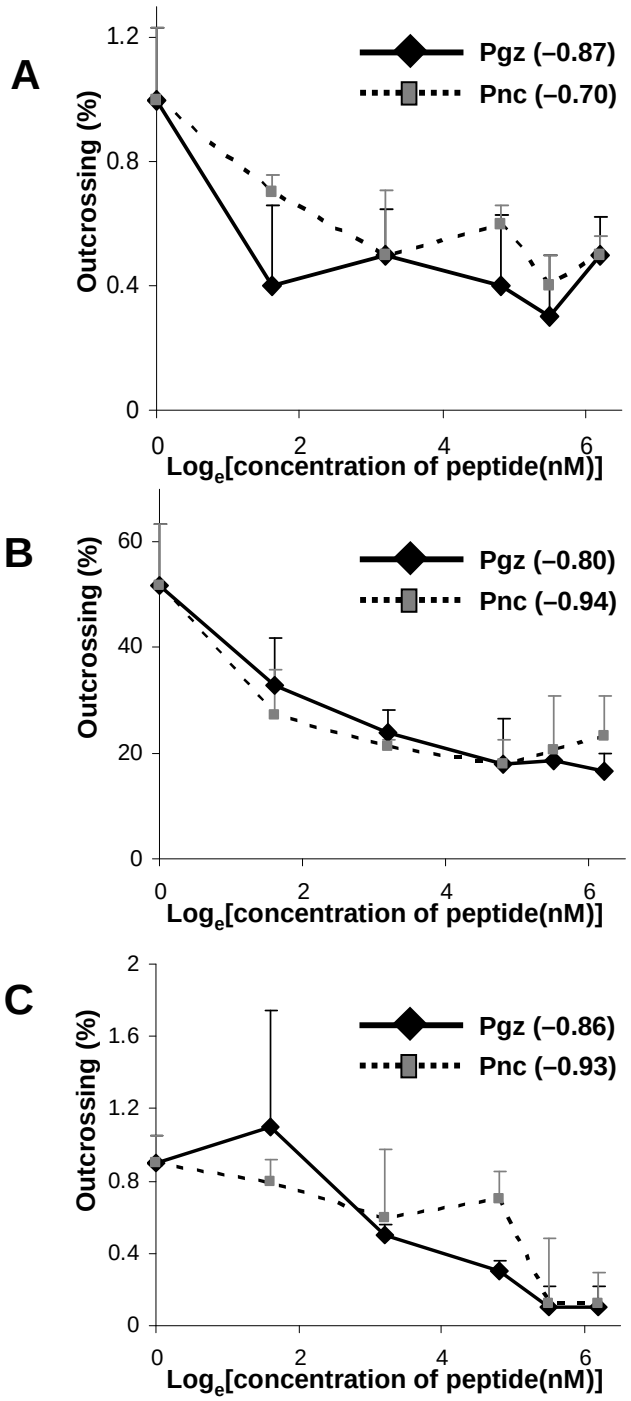

Figure 3.9 Outcrossing fertility of *G. zeae* in the presence of synthetic peptides of *G. zeae* and *N. crassa*. *G. zeae* wild type strain Z-3639 (A), *Δppg1* (B), and *Δpre2* (C) were used for female and GFP-tagged strain, GFPZ-3639 was used for male.

CHAPTER 4 - INHIBITION OF SPORE GERMINATION OF *GIBBERELLA ZEAE* BY PHEROMONE PEPTIDES

Abstract

In heterothallic Ascomycete fungi, pheromones play an important role in fertilization in sexual reproduction by enabling interactions between male and female reproductive cells. In addition, pheromone peptides inhibit conidial germination in *Neurospora crassa* and arrest the G1 phase of the cell cycle in *Saccharomyces cerevisiae*. An α-factor-like pheromone peptide functions in fertilization of *G. zeae*, even though it is a homothallic fungus. The objectives of this study were: (1) to determine if spore germination of *G. zeae* was affected by α-factor-like pheromone peptides produced by *G. zeae* (10 amino acid residues) and *N. crassa* (11 amino acid residues), (2) to determine if the effects are mediated by the *pre2* pheromone receptor, and (3) to determine the efficacy of synthetic pheromone peptides that contain portions of the *N. crassa* and part of the *G. zeae* amino acid sequences. Both pheromone peptides inhibited germination of conidia and ascospores in water (non-nutritional conditions), but not in YEPD medium (nutrient-rich). The *N. crassa* phermone inhibited spore germination much more efficiently than did the *G. zeae* phermone. Synthetic pheromone peptide sequences were produced by the substitution of amino acids from the *N. crassa* peptide into the *G. zeae* peptide. Arginine and lysine residues in the peptides were the most important determinants of the ability to inhibit spore germination. This result suggests that pheromone peptides of *G. zeae* and *N. crassa*, or derivatives therefrom, could be used as fungistatic agents against *G. zeae*, and other filamentous Ascomycetes, and that

the efficacy of the pheromone peptides could be altered by substituting amino acids at critical locations.

Introduction

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is the most important causal agent of Fusarium head blight (FHB, also termed scab) of wheat and barley (McMullen *et al.*, 1997). In addition to direct yield loss, *G. zeae* can reduce grain quality through the production of mycotoxins such as trichothecenes and zearalenone (Desjardins, 2006) that contaminate the grain.

G. zeae produces sexual spores (ascospores) and asexual spores (conidia) for inoculum dispersal (Markell and Francl, 2003). Ascospores are produced in perithecia commonly found on crop residue. These spores are forcibly discharged from the perithecia during periods of high humidity (Sutton, 1982; Trail *et al.*, 2002). Conidia are produced abundantly on sporodochia found on crop residue during warm and moist conditions. Host plant infection is initiated by ascospores or conidia in florets between the anthesis and soft dough stages of kernel development (Schroeder and Christensen, 1963). Initially (Adams, 1921) the anthers were identified as the initial infection site, but since then the hyphae have been found to directly penetrate ovaries or the inner walls of lemmas, paleas, or glumes (Bushnell *et al.*, 2003). Symptoms appear at the point of infection in the form of water-soaked brown spots. The fungus invades rapidly and initially kills the original floret and eventually the entire spikelet. Subsequently, the fungus may spread up and down the rachis of the spike and invade additional spikelets (Parry *et al.*, 1995).

Chemical and biological methods have been used to control FHB, but the development of resistant varieties should provide the most economical disease control (Parry *et al.*, 1995).

Resistance to FHB in wheat is controlled by several quantitative trait loci (QTL) (Bai and Shaner, 2004; Ban and Suenaga, 2000; Snijders, 1990). Although wheat cultivars with good resistance to FHB have been produced (Bai and Shaner, 2004; Gilbert and Tekauz, 2000), none have sufficient resistance to withstand high disease pressure. Similarly, current levels of resistance to FHB in barley are not considered sufficient (Bai and Shaner, 2004).

FHB resistance is classified into two types: Type I resistance is resistance to the initial infection, and Type II resistance is resistance to the spread of the fungus within the spike (Schroeder and Christensen, 1963). Additional resistance types have been described including resistance to kernel infection, tolerance to avoid yield loss, and resistance to toxin accumulation (Mesterhazy, 1995; Miller *et al.,* 1985). The molecular mechanisms of these different types of resistance are not understood. In wheat and barley, Type II resistance is the most common, although the independence of the different types has been questioned (Bai and Shaner, 2004).

Another possible method to combat FHB is through a transgenic approach. Overexpression of plant-derived pathogenesis-related (PR) proteins such as thaumatin-like proteins, chitinase, and glucanase, can provide measurable resistance against this disease, but individual transgenes are insufficient whenever epidemic conditions occur (Anand *et al*., 2003; Chen *et al*., 1999; Pellegrineschi *et al.,* 2001). The most successful transgenic strategy to date has utilized a pathogen-derived gene. The fungal gene *TRI101* encodes the enzyme trichothecene acetyltransferase, which reduces the activity of the mycotoxin deoxynivalenol (DON) by adding an acetyl group to the C3 hydroxyl group (Okubara *et al*., 2002). Transgenic plants expressing *TRI101* exhibited partial resistance to *G. zeae* in a greenhouse test (Okubara *et al.,* 2002). A similar mechanism of detoxification was found for *DOGT1*, a UDP-glycosyltransferase of *A. thaliana* that detoxifies DON by adding a glucose residue to the C3

hydroxyl group. Overexpression of the *DOGT1* gene in *A. thaliana* increases DON resistance (Poppenberger *et al.,* 2003).

Fungal sex pheromone peptides are potential targets for a pathogen-derived transgenic approach because they are secreted intercellular communication signals that may have strong effects on fungal behavior. In other systems, they can affect fungal germination, filamentous growth, chemotaxis, sexual development, and pathogenicity (Kim and Borkovich, 2004; Kim *et al.,* 2002; Kronstad and Staben, 1997; Pöggeler and Kück, 2001; Snetselaar *et al.,* 1996; Spellig *et al.,* 1994). In *Neurospora crassa,* sex pheromones secreted by trichogynes inhibit germination of conidia of the opposite mating type (Bistis, 1983). Pheromone factors of *Saccharomyces cerevisiae* cause cells of the opposite mating type to arrest growth in the G1 phase of the cell cycle just before the initiation of DNA synthesis (Betz *et al.,* 1977; Bücking-Throm *et al.,* 1973; Hereford and Hartwell, 1974). α-factor pheromone of *S. cerevisiae* inhibits appressorium formation of *M. grisea* (Beckerman *et al.,* 1997) and *Glomerella cingulata* (Al-Samarrai *et al.,* 2002). It is unknown whether all these effects are mediated by sex pheromone receptors or by some other mechanism.

The genome of *G. zeae* contains two putative pheromone precursor genes and two putative pheromone receptor genes, which are homologous to those in other filamentous ascomycetes (Chapter 3). One pheromone/receptor pair (*ppg1*/*pre2*) was functional in fertilization and the other pair (*ppg2*/*pre1*) appeared not to be functional. *ppg1* of *G. zeae* produces an α-factor-like pheromone peptide (Pgz) that is similar to the α-factor-like pheromone peptide from *N. crassa* (Pnc). We predicted that the Pgz peptide would inhibit spore germination in *G. zeae* because the Pnc peptide efficiently inhibits conidial germination in *N. crassa*. If that were true, then Pgz might be expressed in transgenic wheat or barley spikes as a novel form of

Type I resistance. The objectives of this study were: (1) to determine if peptide phermones produced by *G. zeae* (Pgz) and *N. crassa* (Pnc) inhibit the germination of conidia and ascospores produced by *G. zeae*, (2) to determine whether *pre2* mediates the action of the peptide phermone(s), and (3) to determine the efficacy of synthetic pheromone peptides in the spore germination process.

Materials and Methods

Fungal strains: *G. zeae* wild type strain, Z-3639 isolated from Kansas (Bowden and Leslie, 1992) and a *pre2*-deletion mutant, *Δpre2*, derived from it were stored as frozen conidial suspensions in 15% glycerol at –70°C. Details of mutant construction by the split marker method were described in Chapter 3.

Synthetic peptides: Synthetic α-factor-like peptides of *G. zeae* (10 amino acid residues) and *N. crassa* (11 amino acid residues) were synthesized by Celtek Peptide Co. (Nashville, TN). Peptide sequences were "WCWWKGQPCW" and "QWCRIHGQSCW" for *G. zeae* and *N. crassa*, respectively. There are five amino acid residues that differ between the two pheromones. The residues of the two peptides were substituted into the other's sequence to assess the importance of each amino acid residue for spore germination inhibition. All ten single amino acid substitutions were synthesized (Table 4.1). Each peptide was dissolved in 2% (v/v) dimethylsulfoxide (DMSO) at 1 mM and stored at 4°C.

Preparation of spores: Z-3639 and *Δpre2* were inoculated on carrot agar and incubated at 24°C with a 12 hr photoperiod under fluorescent light (Leslie and Summerell, 2006). After three days, mycelia were mock-fertilized with 500 µl of an aqueous Tween 60 solution (2.5% v/v) to induce sexual development. Conidia and ascospores were harvested separately. One ml of sterile water or YEPD (3 g yeast extract, 10 g peptone, and 20 g glucose per liter) medium

was added to the plate at three days after induction to harvest conidia. Ten days after induction, each plate was placed upside-down for 24 hours and discharged ascospores collected on the lid of a clean plate. One ml of sterile water was added to the lid to harvest the ascospores. Spore concentrations were adjusted to 10^4 spores/ml.

Spore germination test with synthetic peptides: Germination of spores was checked in four experiments. Three replications were performed for Pgz and Pnc but one experiment was done for the other ten synthetic peptides. (1) The original solution (1mM) was diluted and100 µl of diluted peptide suspensions added to 900 µl of spore suspension (10^4 spores/ml in water) to a final concentration of 0, 1, 10, 20, 40, and 80 µM. The tubes were incubated at 22–24°C on an orbital shaker (120 rpm) and germination of 50 spores was checked microscopically (100×) at 6, 18, 30, 42, 54, 66, and 120 hr. (2) A 15 µl drop of a spore suspension (10^4 spores/ml in water) was placed on a slide glass and 15 µl of peptide solution was added to each drop at the same final concentration as above. (3) 15 µl of spore suspension (10^4 spores/ml in YEPD) was placed on a glass slide and 15 µl of peptide was added to each drop to a final concentration of 0, 100, 250, and 500 µM. (4) 15 µl of YEPD agar (2%) at 60°C was placed on a slide glass and 15 µl of peptide were immediately added to each drop to a final concentration of 0, 100, 250, and 500 µM. After the drop solidified, 2 µl of spore suspension (10^4 spores/ml in water) was placed on it. Spore germination was checked at 24, 48, and 72 hr for the third and fourth experiments.

Antimicrobial properties of peptides: To determine if the synthetic peptides had the same general properties as those of known antimicrobial peptides, the amphiphilic nature, isoelectric point, and net charge, were calculated (DNASTAR-Proteon, DNASTAR Inc., Madison, WI).

To determine if the synthetic peptides have nonspecific antibacterial activity, 1 µl of a bacterial culture (*E. coli* strain JM109; Promega, Madison, WI) grown at 37°C for 18 hrs was inoculated in 200 µl of LB medium containing peptides at a final concentration of 500 µM. The cultures were incubated at 37°C for 18 hrs on an orbital shaker (200 rpm). Bacterial cells were pelleted by centrifugation for 1 min at 8,000 × g and wet biomass compared with that from an untreated culture.

To test whether synthetic peptides have nonspecific antifungal activity, conidia from *M. grisea* strain CP987 (Beckerman *et al*., 1997) grown on oat meal agar were harvested in sterile water and the concentration was adjusted to 10^5conidia/ml. Fifteen µl of the conidial suspension was placed on a glass slide and 15 µl of peptide solution was added to the drop at a final concentration of 100 µM. Conidial germination was checked microscopically (100×) at 48 hrs.

***pre2* gene homologs:** The existence of another receptor with homology to *pre2* was tested in two ways: (1) The *pre2* gene of *G. zeae* (FG02655; Chapter 3) was blasted to the *Fusarium* genome database (http://www.broad.mit.edu/annotation/fungi /fusarium/) using blastn and blastp. (2) Southern hybridizations were done with the *pre2* gene as a probe to genomic DNA of Z3639 using Gene Images AlkPhos direct Labelling and Detection System (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions.

Results

Germination inhibition by phermone peptides and their derivatives in water: There was no difference in germination inhibition between the tests done in 1 ml (0.2% DMSO) and 30 µl (1.0% DMSO) volumes. Therefore, subsequent experiments were done in 30 µl of 1.0% DMSO on a glass slide. The reaction of both ascospores and conidia for the peptides were the same, and there was no difference between Z-3639 and *Δpre2* (Table 4.2).

At 1 μM, all peptides completely inhibited germination at 6 hr. Neither Pgz nor Pnc completely inhibited germination at 18 hr. Derivatives Pgz-S1 and Pgz-S3 performed similarly to Pgz. Derivatives Pgz-S2, Pgz-S4, and Pgz-S5 completely inhibited germination for at least 30 hr after treatment. For these three peptides, even after germination began, the percentage of germination was low and growth was slow. The efficacy of Pnc-S2 and Pnc-S4 was less than that of other derivatives of Pnc. Pnc-S1, Pnc-S3, and Pnc-S5 had effects similar to those of Pgz-S2, Pgz-S4, and Pgz-S5. Pnc-S3 was superior to all of the other peptides, and could completely inhibit germination for at least 54 hours (Table 4.2).

The efficacy of peptides at 10 μM differed from that at 1 μM. Pnc completely inhibited germination for 5 days, but Pgz only partially inhibited germination at 18 hr. Pgz-S1 was not as effective as Pgz, but Pgz-S2, Pgz-S3, Pgz-S4, and Pgz-S5 were more effective than Pgz. Pnc-S3 performed as well as Pnc. Ten μM Pnc, Pnc-S1, Pnc-S3, Pnc-S4, and Pnc-S5 completely inhibited germination for at least 42 hours after treatment. At 10 μM of Pnc or its derivatives, the efficacy of Pnc and Pnc-S3 were the highest and that of Pnc-S2 the lowest. The pattern of efficacy at higher peptide concentrations was similar to that observed at 10 μM.

Germination inhibition with synthetic peptide in YEPD medium: The pattern of inhibition in YEPD media (either liquid or solid) was different from that in water (Table 4.3). Germination was not completely inhibited even at 500 μM peptide, except for Pnc-S4. Only 500 μM of Pnc-S4 completely inhibited germination in YEPD. Pgz-S2, Pnc, Pnc-S1, and Pnc-S3 did not reduce germination but did reduce growth rates of germ tubes (Table 4.3).

Antimicrobial properties of peptides: In general, most antimicrobial peptides are amphiphilic, positively charged (+2 to +9) and highly cationic (Jenssen *et al.,* 2006). However, Pnc and Pgz are short peptides that probably cannot form the helical shapes that confer the

amphiphilic properties on many antimicrobial peptide molecules. The net charges of Pnc (+2) and Pgz (+1) were relatively small, but both had the same isoelectric point (Table 4.4). Switching the tryptophan and arginine residues in position 2 between Pgz and Pnc changed both the isoelectric point and the net charge (Pgz-S2 and Pnc-S2; Table 4.4) as did switching the lysine and histidine residues at position 4 (Pgz-S4 and Pnc-S4; Table 4.4).

No evidence was found for broad spectrum antimicrobial activity by any of the 12 peptides examined. The biomass of bacterial cells treated with 500 μM of synthetic peptide was not significantly different from that of an untreated control. Neither conidial germination nor germ tube elongation of *M. grisea* were inhibited by 100 μM pheromones or synthetic peptides.

***pre2* gene homologs:** Blastp and blastn searches with the putative *pre2* gene (FG02655) did not identify any homologs of *pre2* in the *F. graminearum* genome at significance levels below E-value of 1e-3. Blastn did not identify any homolog under E-value of 1e-1 but blastp showed one homolog (FG00333) under E-value of 1e-1. FG00333 has not been characterized, but the protein it encodes contains seven transmembrane domains, as does *pre2*.

For Southern hybridizations, *G. zeae* strain, Z-3639 was digested with seven different endonucleases (BamHI, ClaI, XhoI, SalI, EcoRI, and HindIII) and the *pre2* gene was used as a probe to estimate the copy number of *pre2* gene homologs. *G. zeae* has only one copy of *pre2* (a strong band) but there was a second weak band (Figure 4.1), whose presence is consistent with the hypothesis that a homolog of *pre2* exists in *G. zeae* even though a corresponding sequence was not identified in the *F. graminearum* database.

Discussion

α-factor-like pheromone peptides and related substituted synthetic peptides of *G. zeae* and *N. crassa* inhibited spore germination of *G. zeae* in a concentration, time, growth conditions

and peptide dependent manner. At a concentration of 1 μM, most peptides could inhibit germination of both conidia and ascospores for at least 18 hr. At 20 μM, most peptides could inhibit germination for at least 42 hr. At 80 μM, most peptides inhibited germination for five days, the longest time tested. In addition, fungistatic effects often persisted as reduced germination rates long after the complete inhibition of germination was lost. Pgz, the native pheromone from *G. zeae*, was one of the least effective peptides in blocking the germination of *G. zeae* spores. In contrast, Pnc, the pheromone from *N. crassa*, was one of the most effective. At the lowest tested concentration (1 μM), the most effective peptide was Pnc-S3, which completely inhibited germination for at least 54 hr.

The complete inhibition of germination of both ascospores and conidia of *G. zeae* by low concentrations of pheromone peptides suggests that pheromones peptides or their derivatives might be exploited for disease control. Ascospores and conidia function as the primary inoculum in *G. zeae* (Brown *et al*., 2001; Shaner, 2003) so inhibiting or delaying their germination might prevent penetration of plants by the pathogen. For example, a transgenic plant producing pheromone peptides in the spikes could display a novel form of Type I resistance to initial infection. This Type I resistance could be combined with existing Type II resistances to produce cultivars with higher overall resistance. Alternatively, the peptides might be useful fungistatic compounds for chemical control or delivery through *in situ* synthesis by a biological control agent. The feasibility of any of these strategies would depend on many factors including the stability of the peptides and the ability to deliver the peptide to the infection court at the right time at an effective concentration.

The lowest effective concentrations of pheromones and synthetic peptides demonstrated in this study were higher than reported in some other fungi. Approximately 70 nM of α-factor

pheromone of *Candida albicans* effects morphological change in *C. albicans* (Dignard and Whiteway, 2006), but 1.7 nM of α-factor pheromone of *S. cerevisiae* is enough to arrest the G1 phase of *S. cerevisiae* (Raths *et al.*, 1988). In this study, the lowest effective concentration was 1 μM, but this is certainly an overestimate because lower concentrations were not tested. Further work is needed to establish the lowest effective concentrations of these peptides.

The pattern of specificity of germination inhibition was complex. One μM Pgz inhibited germination of *G. zeae*, but even 100 μM had no effect on germination of conidia of *M. grisea* and 500 μM had no effect on growth of *E. coli*. Unexpectedly, Pnc was more effective at inhibiting spore germination of *G. zeae* than the native pheromone Pgz. These results suggest that the germination inhibition effect may be the result of specific interactions rather than general antimicrobial effects.

The difference in efficacy of Pgz and Pnc was investigated by making single amino acid substitutions in Pgz and Pnc. Pgz-S2 was strongly enhanced for germination inhibition and the efficacy of Pnc-S2 decreased, suggesting that the arginine residue (strongly basic) at position 2 may be important for efficacy. In both cases, the substitution of a more basic residue increased the efficacy of spore germination inhibition. Even more effective germination inhibitors might be discovered by a more thorough analysis of similar peptides.

The mechanism of spore germination inhibition by pheromone peptides in *G. zeae* is independent of the *pre2* pheromone receptor. Previously we demonstrated that the *pre2* gene product in *G. zeae* interacts with the α-factor-like pheromone of *G. zeae* (Chapter 3). Theoretically, a *Δpre2* mutant should not be affected by α-factor-like pheromone peptides (Pgz or Pnc) because it does not have the receptor required to interact with the pheromone peptide.

However, *Δpre2* spore germination was inhibited at the same peptide concentrations as was the wild type strain, Z-3639.

One possible explanation is that the pheromone peptides inhibit spore germination as antifungal peptides independent from interactions with any pheromone receptors. The antifungal activity of small peptides has been described in a number of organisms (Jenssen *et al.*, 2006). Antifungal peptides exert their effects through the lysis of cellular membranes or interference with essential cellular components. The mode of action of pheromone peptides is unlikely to be related to that of antifungal peptides for several reasons. First, pheromone peptides do not have general antifungal protein properties such as being amphiphilic or highly positively charged (De Lucca and Walsh, 1999; Table 4.4). Second, peptide pheromones are fungistatic, while antimicrobial peptides are fungicidal. Third, peptide pheromones have a narrow spectrum of activity but antimicrobial peptides have broad spectrum activity, *e.g.*, neither Pgz nor Pnc nor any of their derivatives affected growth of *E. coli* or germination of *M. grisea*. Fourth, no inhibition by pheromone peptides occurred in nutrient-rich medium.

An alternative explanation for these results is that *G. zeae* has a second receptor for the pheromone. If the second receptor has a conserved binding site, then this region might have significant homology with *pre2*. However, no significant homolog of *pre2* was found in the *F. graminearum* database. Southern hybridization showed that *G. zeae* strain, Z-3639 has one strong band (presumably *pre2*) and one weak band when probed with *pre2* DNA (Figure 4.1). The weak band could be a second receptor for *ppg1*. Additional work is needed to identify and characterize this potential second receptor.

The inhibitory effect of peptide pheromones on spore germination usually was not observed in liquid and solid YEPD medium, although several peptides did retard germ tube

elongation. Only Pnc-S4, in which the histidine residue (weakly basic) of Pnc was substituted by lysine (basic), could inhibit spore germination on YEPD. These results suggest that the mechanism by which germination is inhibited by pheromones is not operating at high nutrient conditions. Cross-talk between pheromone-sensing and nutrient-sensing pathways has been reported in other fungi (Lengeler *et al*., 2000). Haploid cells of *S. cerevisiae* mate in rich medium to form diploid cells that only later sporulate under conditions of nitrogen and carbon starvation. The glucose-sensing pathway also regulates the downstream pheromone pathway in *Schizosaccharomyces pombe* (Lengeler *et al.*, 2000). The unusual behavior of Pnc-S4 occurred only at high concentrations (500 μM), but it suggests that the interactions of this peptide are less affected by starvation.

There is a simple model to explain the cross-talk between germination inhibition by pheromone peptides and repression by nutrients (Figure 4.2). In the first model (Figure 4.2A), there is a second receptor in addition to the *pre2* receptor. Pheromone peptides compete with nutrient molecules for binding to the second receptor. Binding of the pheromone peptide to the second receptor results in germination inhibition, while nutrient binding enables vegetative growth and inhibits sexual reproduction. In the second model, there is an additional receptor only for nutrient-sensing. Binding of the nutrient to the third receptor enables vegetative growth and inhibits sexual reproduction and germination by pheromone peptides. One candidate for a nutrient-sensing receptor in both models is the glucose-sensing receptor that regulates sexual reproduction in *S. pombe*. The *G. zeae* genome database contains one copy (FG05006) of a glucose-sensing receptor. Characterization of this gene might help test the models.

In summary, this study showed that pheromone peptides can efficiently inhibit spore germination by *G. zeae*, and that novel peptides with higher efficacy can be synthesized by

substituting amino acids into these pheromone peptides. The inhibition mechanism through which these peptides act has not been characterized, but it is affected by nutrients and is independent of the *pre2* pheromone receptor.

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Table 4.1 Amino acid residues of pheromone peptides and synthetic peptides

Name	Amino acid residues[b] (M.W.)[c]	Name	Amino acid residues (M.W.)
Pgz[a]	WCWWKGQPCW (1380)	Pnc	QWCRIHGQSCW (1405)
Pgz-S1	**Q**WCWWKGQPCW (1548)	Pnc-S1	WCRIHGQSCW (1316)
Pgz-S2	WC**R**WKGQPCW (1390)	Pnc-S2	QWC**W**IHGQSCW (1474)
Pgz-S3	WCW**I**KGQPCW (1347)	Pnc-S3	QWCR**W**HGQSCW (1517)
Pgz-S4	WCWW**H**GQPCW (1429)	Pnc-S4	QWCRI**K**GQSCW (1435)
Pgz-S5	WCWWKGQ**S**CW (1410)	Pnc-S5	QWCRIHGQ**P**CW (1454)

[a] Pgz and Pnc are α-factor-like pheromone peptides of *G. zeae* and *N. crassa*, respectively. Identical amino acid residues between Pgz and Pnc are underlined.

[b] Amino acid residues in boldface type between Pgz and Pnc were substituted.

[c] Molecular weight

Table 4.2 Germination tests with synthetic peptides in water.

Concentration of peptide	peptide	Germination of spores [a]						
		6 hrs	18 hrs	30 hrs	42 hrs	54 hrs	66 hrs	5 days
0 μM	control	++++	++++	++++	++++	++++	++++	++++
1 μM	Pgz	–	+++	+++	+++	++++	++++	++++
	Pgz-S1	–	++++	++++	++++	++++	++++	++++
	Pgz-S2	–	–	–	–	+	+	++
	Pgz-S3	–	++++	++++	++++	++++	++++	++++
	Pgz-S4	–	–	–	+	+	+	+
	Pgz-S5	–	–	–	–	+	+	+
	Pnc	–	+++	++++	++++	++++	++++	++++
	Pnc-S1	–	–	–	–	+	++	++
	Pnc-S2	–	–	++	++	+++	+++	+++
	Pnc-S3	–	–	–	–	–	+	+
	Pnc-S4	–	++	++	++	+++	+++	+++
	Pnc-S5	–	–	–	–	++	++	++
10 μM	Pgz	–	++	++	+++	++++	++++	++++
	Pgz-S1	–	+++	+++	+++	++++	++++	++++
	Pgz-S2	–	–	–	–	+	+	+
	Pgz-S3	–	–	–	–	+	++	++
	Pgz-S4	–	–	–	+	+	+	+
	Pgz-S5	–	–	–	–	+	+	+
	Pnc	–	–	–	–	–	–	–
	Pnc-S1	–	–	–	–	–	–	++
	Pnc-S2	–	–	++	++	+++	+++	+++

	Pnc-S3	–	–	–	–	–	–	–
	Pnc-S4	–	–	–	–	++	++	++
	Pnc-S5	–	–	–	–	++	++	++
20 μM	Pgz	–	–	++	++	++	++	++
	Pgz-S1	–	–	++	++	++	++	++
	Pgz-S2	–	–	–	–	–	–	–
	Pgz-S3	–	–	–	–	+	++	++
	Pgz-S4	–	–	–	+	+	+	+
	Pgz-S5	–	–	–	–	–	–	–
	Pnc	–	–	–	–	–	–	–
	Pnc-S1	–	–	–	–	–	–	–
	Pnc-S2	–	–	–	++	+++	+++	+++
	Pnc-S3	–	–	–	–	–	–	–
	Pnc-S4	–	–	–	–	–	–	–
	Pnc-S5	–	–	–	–	+	++	++
40 μM	Pgz	–	–	++	++	++	++	++
	Pgz-S1	–	–	–	++	++	++	++
	Pgz-S2	–	–	–	–	–	–	–
	Pgz-S3	–	–	–	–	+	++	++
	Pgz-S4	–	–	–	+	+	+	+
	Pgz-S5	–	–	–	–	–	–	–
	Pnc	–	–	–	–	–	–	–
	Pnc-S1	–	–	–	–	–	–	–
	Pnc-S2	–	–	–	++	++	+++	+++
	Pnc-S3	–	–	–	–	–	–	–

	Pnc-S4	–	–	–	–	–	–	–
	Pnc-S5	–	–	–	–	+	+	+
80 μM	Pgz	–	–	++	++	++	++	++
	Pgz-S1	–	–	–	–	+	+	+
	Pgz-S2	–	–	–	–	–	–	–
	Pgz-S3	–	–	–	–	+	+	+
	Pgz-S4	–	–	–	–	–	–	–
	Pgz-S5	–	–	–	–	–	–	–
	Pnc	–	–	–	–	–	–	–
	Pnc-S1	–	–	–	–	–	–	–
	Pnc-S2	–	–	–	–	–	++	++
	Pnc-S3	–	–	–	–	–	–	–
	Pnc-S4	–	–	–	–	–	–	–
	Pnc-S5	–	–	–	–	–	+	+

[a] Asocospores and conidia were affected at the same level and there was no difference between wild type strain Z-3639 and *pre2*-deletion mutant (*Δpre2*). Minus (–) represents no spore germination; + less than 25%; ++ 25-49%; +++ 50-74%; ++++ 75-100% germination. For the test, a 15 μl drop of spore suspension (10^4 spores/ml in water) was placed on a slide glass and 15 μl of peptide solution was added to each drop at the final concentration.

Table 4.3 Germination test with synthetic peptides in YEPD medium.

Concentration of peptide	Peptide	Germination of spores[a]		
		24 hrs	48 hrs	72 hrs
0 μM	Control	++++	++++	++++
100 μM	Pgz	++++	++++	++++
	Pgz-S1	++++	++++	++++
	Pgz-S2	+++	++++	++++
	Pgz-S3	++++	++++	++++
	Pgz-S4	++++	++++	++++
	Pgz-S5	++++	++++	++++
	Pnc	+++	++++	++++
	Pnc-S1	++++	++++	++++
	Pnc-S2	++++	++++	++++
	Pnc-S3	+++	++++	++++
	Pnc-S4	++	+++	++++
	Pnc-S5	++++	++++	++++
250 μM	Pgz	++++	++++	++++
	Pgz-S1	++++	++++	++++
	Pgz-S2	++	+++	+++
	Pgz-S3	++++	++++	++++
	Pgz-S4	++++	++++	++++
	Pgz-S5	++++	++++	++++
	Pnc	++	+++	++++
	Pnc-S1	++	+++	++++
	Pnc-S2	++++	++++	++++

	Pnc-S3	++	+++	++++
	Pnc-S4	+	++	+++
	Pnc-S5	++++	++++	++++
500 μM	Pgz	++++	++++	++++
	Pgz-S1	++++	++++	++++
	Pgz-S2	+	++	++
	Pgz-S3	++++	++++	++++
	Pgz-S4	++++	++++	++++
	Pgz-S5	++++	++++	++++
	Pnc	+	++	++
	Pnc-S1	+	++	++
	Pnc-S2	++++	++++	++++
	Pnc-S3	+	+	+
	Pnc-S4	–	–	–
	Pnc-S5	++++	++++	++++

[a] Asocospores and conidia were affected at the same level and there was no difference between wild type strain Z-3639 and *pre2*-deletion mutant (*⊿pre2*). Minus (–) represents that no spore germinates, and plus (+) represents that almost 100% spores germinate. The number of plus represents the length of germ tubes compared to that of wild typ (+ smaller than 1/4; ++ 1/4 - 2/4; +++ 2/4 - 3/4; ++++ 3/4 - 4/4). For the test, 15 μl of spore suspension (10^4 spores/ml in YEPD) was placed on a slide glass and 15 μl of peptide was added to each drop at the final concentration.

Table 4.4 Isoelectric point and net charge of synthetic peptides

Peptide	Pgz	Pgz -S1	Pgz -S2	Pgz -S3	Pgz -S4	Pgz -S5	Pnc	Pnc S-1	Pnc -S2	Pnc -S3	Pnc -S4	Pnc -S5
IP[a]	8.3	8.3	9.1	8.3	6.9	8.3	8.3	8.3	6.9	8.3	9.1	8.3
NC[b]	+1	+1	+2	+1	+1	+1	+2	+2	+1	+2	+2	+2

[a] Isoelectric point.

[b] Net charge

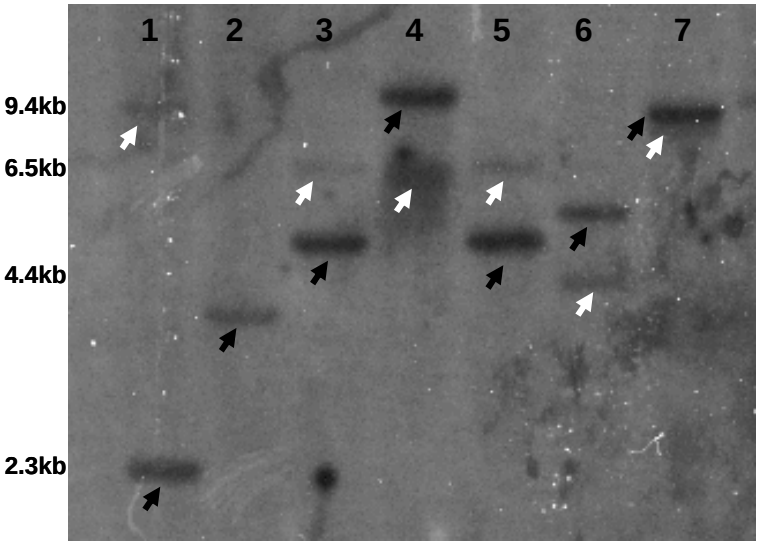

Figure 4.1 Southern hybridization to confirm the copy number of *pre2* gene. *G. zeae* wild type strain Z3639 was digested with seven different enzymes (BamHI, ClaI, XhoI, XbaI, SalI, EcoRI, and HindIII) and the *pre2* gene was used as a probe. Black and white arrows indicate the expected fragment and another homolog of *pre2* gene, respectively.

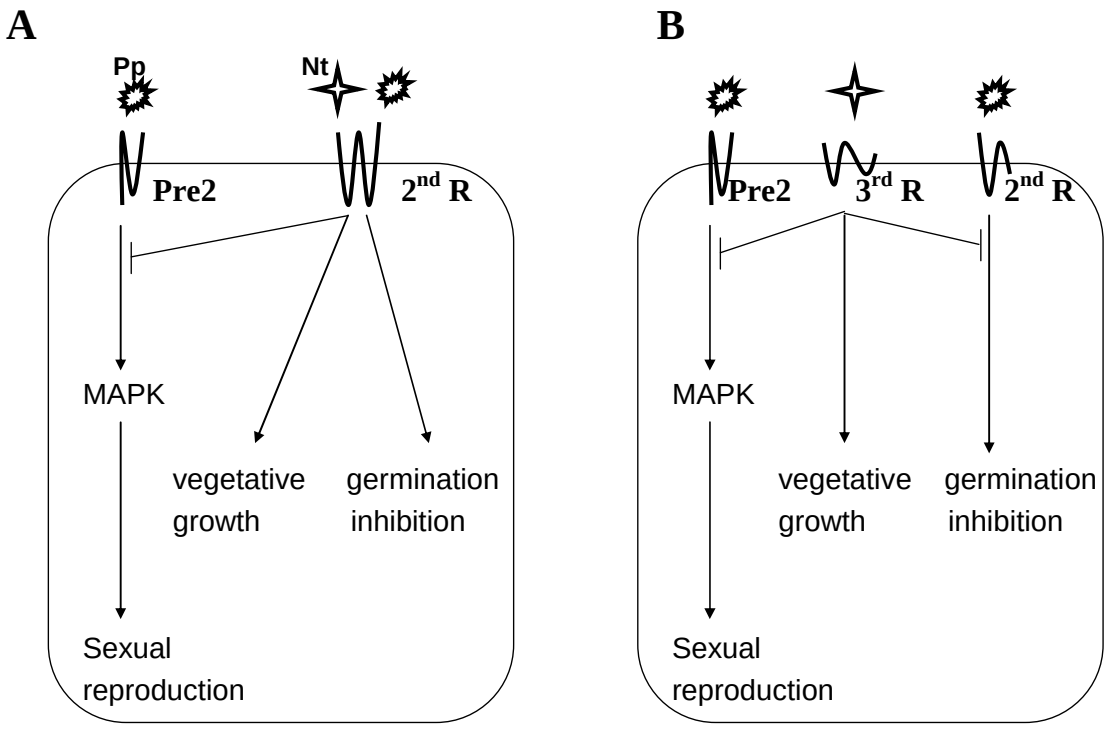

Figure 4.2 Models for germination inhibition by pheromone peptides. (A) Pheromone peptide (Pp) and nutrient (Nt) compete to bind to the 2nd receptor (2nd R). Binding of Pp to the 2nd R triggers germination inhibition, while binding of nutrient to the 2nd R trigger vegetative growth and block sexual reproduction. (B) There are three receptors. The 2nd receptor binds to Pr to trigger germination inhibition. The 3rd receptor binds to only nutrient and this binding triggers vegetative growth and blocks sexual reproduction and germination inhibition.