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A Two-Component Regulatory System for Self/Non-Self Recognition in Ustilago maydis

Bernd Gillissen, Jörg Bergemann, Claus Sandmann, Birgit Schroeer, Michael Bölker, and Regine Kahmann
Institut für Genbiologische Forschung Berlin GmbH
Ihnestr. 63
D-1000 Berlin 33
Federal Republic of Germany

Summary

In U. maydis the multiallelic *b* locus controls sexual and pathogenic development. In the *b* locus a gene coding for a regulatory protein had been identified, and it was suggested that the interaction of two *b* polypeptides specified by different alleles programs sexual development in this fungus. We now demonstrate the existence of a second regulatory gene in the *b* locus. We term this gene *bW* and refer to the former as the *bE* gene. Both genes exist in many alleles. Although unrelated in primary sequence, both genes are similar in their overall organization. The gene products display allele-specific variability in their N-terminal domains, show a high degree of sequence conservation in the C-terminal domains, and contain a homeodomain-related motif. Genetic evidence is provided to show that the pair of *bE* and *bW* polypeptides encoded by different *b* alleles is the key regulatory species.

Introduction

The basidiomycete fungus Ustilago maydis is the causative agent of corn smut disease. Its life cycle is characterized by two stages. The fungus can be propagated on defined media as haploid sporidia, which grow as yeast do and are nonpathogenic. When two compatible strains fuse, the pathogenic dikaryon is formed, which requires the host plant for sustained growth. Characteristic symptoms of the disease are galls or tumors (Tum^+ phenotype) that can develop on all green parts of the plant. Formation of the dikaryon is associated with a change in morphology from the yeast-like to the filamentous form. Dikaryon formation can be visualized on complete media to which charcoal has been added; the long filaments appear as white fuzziness (Fuz^+ phenotype) (for reviews see Christensen, 1963; Banuett and Herskowitz, 1988). The mating reaction and all stages of subsequent development are controlled by the two mating type loci *a* and *b*. For a successful mating reaction, partners must carry different alleles at the *a* and the *b* locus. The *a* locus exists in two alleles, *a1* and *a2*, and is thought to control the cell fusion step. Together with *b*, the *a* locus is also responsible for the maintenance of the filamentous form (Rowell and DeVay, 1954; Holliday, 1961; Puhalla, 1968, 1970; Banuett and Herskowitz, 1989). Elsewhere we provide evidence that the *a* locus encodes the structural genes for mating pheromones and their receptors (Bölker et al., 1992). The *b* locus regulates the steps in sexual development that occur after fusion of haploid strains. The locus is multiallelic; 33 strains with different *b* alleles have been isolated from nature (Rowell and DeVay, 1954; Puhalla, 1968; DeVay, quoted in Wong and Wells, 1985). It is one of the most intriguing and fascinating aspects of the U. maydis life cycle that all possible pairwise combinations of haploid strains that carry different *b* alleles produce the filamentous dikaryon, which is infectious. Filamentous growth and pathogenic development are not initiated, however, if cells carry identical *b* alleles (see Banuett and Herskwitz, 1989).

The molecular analysis of the *b* locus (Kronstad and Leong, 1989, 1990; Schulz et al., 1990) has provided insight into the possible mechanisms that allow cells to discriminate between identity and nonidentity at *b*. The sensing of identity or nonidentity at *b* occurs intracellularly and does not require the mating process itself. When diploid strains heterozygous for *a* and homozygous for *b* are transformed with DNA encoding a *b* gene from a different allele, transformants switch from yeast-like to filamentous growth and become pathogenic. Furthermore, haploid strains become pathogenic (albeit less severely) when a different *b* allele is introduced by transformation. The *b* locus encodes a polypeptide of 473 amino acids. *b* polypeptides derived from different alleles show substantial variation in their N-terminal domains, while the C-terminal domains display a high degree of amino acid conservation. The finding of a homeodomain-related motif in the constant region of the *b* polypeptides (Schulz et al., 1990) has led us to propose that the *b* polypeptides are regulatory proteins that fulfill different regulatory functions depending on their combinatorial interactions: homodimers could be repressors, or heterodimers could function as activators for a specific set of genes.

In this paper we provide evidence that the *b* locus encodes a second regulatory polypeptide termed *bW*. We designate the *b* genes described previously as *bE* genes. Both genes exist in many alleles. We demonstrate that it is the pairwise combination of *bW* and *bE* polypeptides derived from different alleles of the *b* locus that turns on sexual development.

Results

The *b* Gene Can Be Deleted without Affecting Mating Specificity

To distinguish between the proposed repressor or activator models, we have generated a derivative of *b2* by deleting almost the entire open reading frame (ORF) of the *b2* gene except for the N-terminal 10 amino acids (Figure 1, "b2-del 20"). The *b2*-del20 construct was introduced in *b1* (FB1) and *b2* (FB2) strains by transformation, and strains were selected in which the resident allele had been replaced (see the Experimental Procedures). Such strains (RK1722 and RK1723) were viable and had no discernible phenotype. When injected into plants as pure cultures they were nonpathogenic, thus ruling out that the *b* homodimer functions as repressor for pathogenic development.

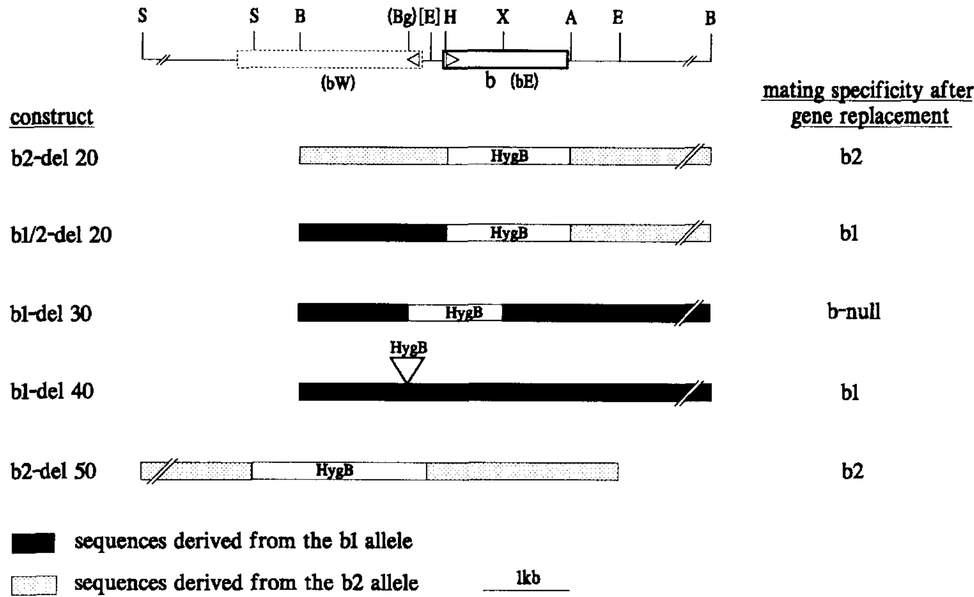


Figure 1. Schematic Representation of DNA Fragments Used for Gene Replacements

The upper line represents a restriction map of the *b2* locus; only sites used for cloning are indicated (S, SalI; B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; X, XbaI; A, ApaLI). The (Bg) site is present only in *b1*; the [E] site is present only in *b2*. Closed boxes represent DNA from the *b1* locus; stippled boxes indicate sequences originating from *b2*. The *b* gene (later termed *bE*) is indicated by an open bar, with the arrow indicating the direction of transcription. The *bW* gene is indicated by a bar with broken lines. The deletion derivatives are drawn to scale with respect to the *b* locus shown in the top line. HygB indicates that a cassette conferring resistance to hygromycin B in U. maydis was inserted at the indicated position; this cassette is not drawn to scale. The fragments shown were isolated from respective plasmids and were used for transformation and gene replacement in *b1* and *b2* strains. *b2* mating specificity indicates that the respective strain can mate with *b1* but not with *b2* strains. *b1* mating specificity indicates that the strain can mate with *b2* and not with *b1* strains. b-null indicates that the strain shows no mating activity.

To determine mating activity of the strains, we performed mating reactions with *b1* and *b2* strains carrying the opposite *a* allele both on charcoal nutrient plates and in planta. The results are summarized in Figures 1 and 3. Unexpectedly, both strains were still able to mate and furthermore displayed mating specificity of *b2*, that is, they were able to mate with *b1* but not with *b2* strains. This result was impossible to reconcile with any of the models

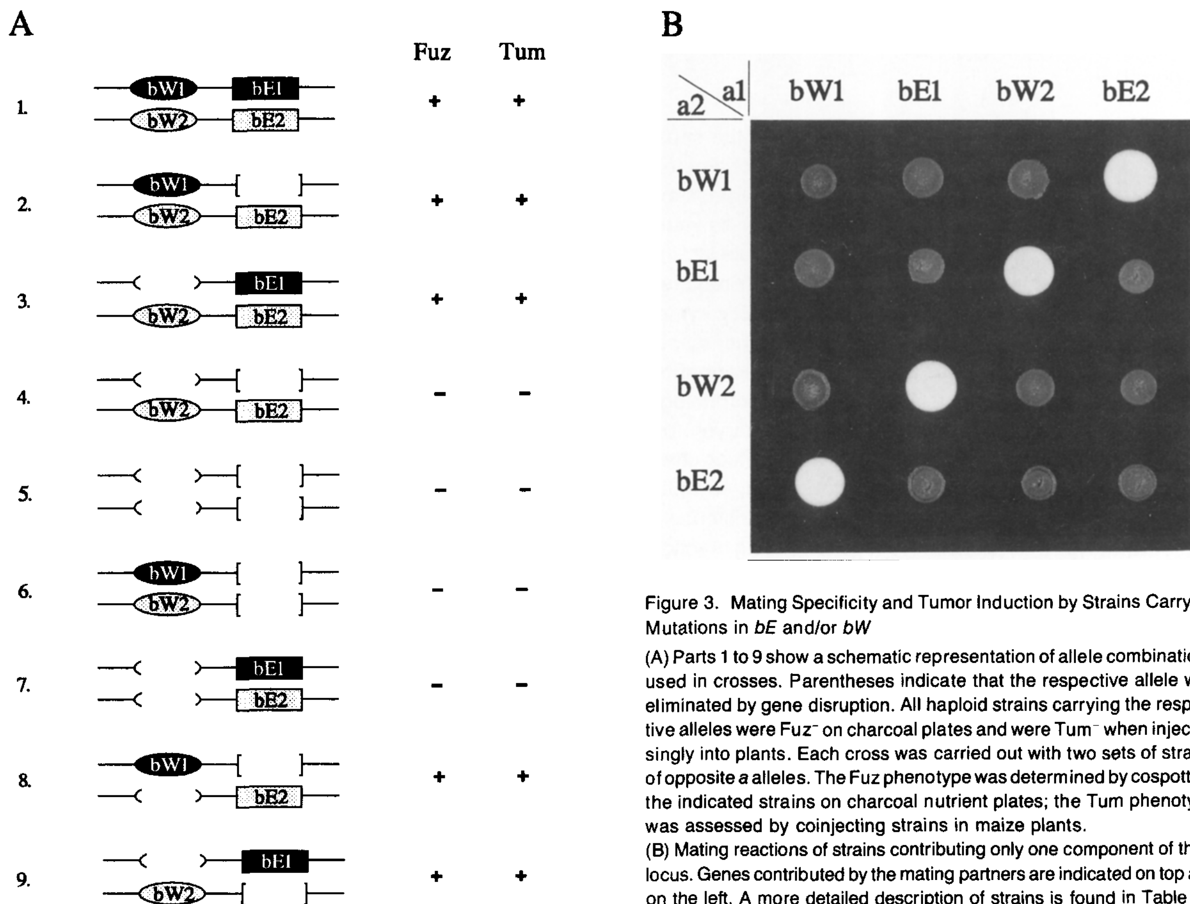
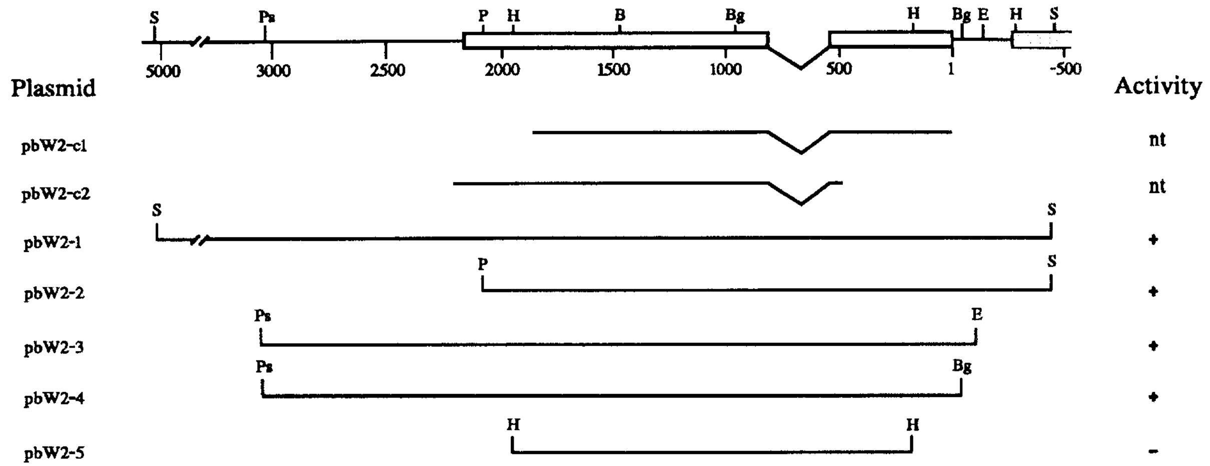


Figure 3. Mating Specificity and Tumor Induction by Strains Carrying Mutations in *bE* and/or *bW*

(A) Parts 1 to 9 show a schematic representation of allele combinations used in crosses. Parentheses indicate that the respective allele was eliminated by gene disruption. All haploid strains carrying the respective alleles were Fuz⁻ on charcoal plates and were Tum⁻ when injected singly into plants. Each cross was carried out with two sets of strains of opposite *a* alleles. The Fuz phenotype was determined by cospotting the indicated strains on charcoal nutrient plates; the Tum phenotype was assessed by coinjecting strains in maize plants.

(B) Mating reactions of strains contributing only one component of the *b* locus. Genes contributed by the mating partners are indicated on top and on the left. A more detailed description of strains is found in Table 1.

A



B

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       <--
-264  GACATTGTGATGATGTTGCAGAGATAGAAGATCTTTTGTTAGAGGCTAAAGGTGGGAGTGCGGTTGAAGAGCGGTGTCGGCGCAAAGAAATGAATGTGTGCACAAAGAAAGGAATTTTGC  -145
        EcoRI                                                                                        BglII
-144  AGACGAATTCGGCCTCAGCACAGGTTCTTTGTTTTCCATCACCATCCACTCAAGTCCCCCTTTTGTCCCCCCAAGTCTCTTCCCAACATGACAGATCTTGAATGTTTCTCCGAGATTTTG  -25
                                -->        1                                                   5
-24   TATTTGACCTCTCAGATCAGAGCGATGCTACCGCCACTGCCAAGAATCTCACAAACCGCACCAAGACCGACCCGTTTCTTTCCACTTAGCCTAGAGAGTCCGAACCAACTGACCCTCAGC  96
                              M  L  P  P  L  P  R  I  S  Q  T  A  P  R  P  T  R  F  F  P  L  S  L  E  S  P  N  Q  L  T  L  S
                                                HindIII
97    CACGAATTGTCGGGCCACGGGGTGAACGGTTCCTATCATGAAGCACTCATCAAGCTTTTCTTGGGACATTTGAACGAACTACACATAGGGTGCCAAGCTCAGTACGAGAGAGTGTTCGCG  216
      H  E  L  S  G  H  G  V  N  G  S  Y  H  E  A  L  I  K  L  F  L  G  H  L  N  E  L  H  I  G  C  Q  A  Q  Y  E  R  V  F  A
217   ATATGGCAACAAGAAAATTTATACGAGGAGGCTTATGATCAGGCGTTCAGAAATCTGCTCAAGCATGTATTCTCCACACGATCACGAAAGATGTGGTACATGCTTCTTAAAGAGGCTAGC  336
      I  W  Q  Q  E  N  L  Y  E  E  A  Y  D  Q  A  F  R  N  L  L  K  H  V  F  S  T  R  S  R  K  M  W  Y  M  L  L  K  E  A  S
337   AAGTACACTCAAAGGGGGTCTTCTCCCCCTGAACCCCACGATGATGATGTCTCCTACGAAGGAGCTCCCCTCAAGACTGGTCGTGGACACGACTCCGAAGCAGTCCGAATCCTGGAGCAA  456
      K  Y  T  Q  R  G  S  S  P  P  E  P  H  D  D  D  V  S  Y  E  G  A  P  L  K  T  G  R  G  H  D  S  E  A  V  R  I  L  E  Q
                                              3
457   GCCTTCAAACATTCACCAAACATCACCCCAGCCGAGAAGTTCCGACTTTCAGAGGTCACTGGACTCAAACCAAAGCAAGTCACTATCTGGGTGAGTGCATGATCTCTCCACGACACAGGT  576
      A  F  K  H  S  P  N  I  T  P  A  E  K  F  R  L  S  E  V  T  G  L  K  P  K  Q  V  T  I  W  ·························
577   CACACCTCAATTTTGTCGTGGTTCGTGGTTCGTGGTCATCTTCCTTCTAATGATGAGATTTTTGGATCTTTTTGACGGGCTGCGCACAAAGGGGAGATCCCTTTGACGATCATATGCCAT  696
      ·····················································270 bp intron·····················································
697   GCAGATTGTAGACTTTTGCTGAAGCCCTCTGTCGCCAAAATGTAACCACTGGATTCTCAGGATCTTTAGTATCAAGTATACTAATTTGTTGGCTTTTTTTGCTCGTCCCTCATATTGCAG  816
      ························································································································
816   TTTCAGAACCGCCGTAACCGGAAAGGGAAGAAGAACCTCAACGTGGAGCCTACGGAATCAACCCAACCAGATCTGTCGCCTTCCAGACACGAGTCACCTCCTCCCTCATCGCCCAGTCGC  936
      F  Q  N  R  R  N  R  K  G  K  K  N  L  N  V  E  P  T  E  S  T  Q  P  D  L  S  P  S  R  H  E  S  P  P  P  S  S  P  S  R
937   GACTTTACCCTTTCCGAAAAGAAGCGCAAATCATACGGCGTGTTGGGTCGTTCTTCCCCCGATTGCACCGACCCCGACTCTGACTCGCCCTCGTCAAGCCTGAAGAAACCTCGGGTGTCA  1056
      D  F  T  L  S  E  K  K  R  K  S  Y  G  V  L  G  R  S  S  P  D  C  T  D  P  D  S  D  S  P  S  S  S  L  K  K  P  R  V  S
              <        6
1057  AGCGTATGTTCGAAACTAAGCGATGGCTCCTCTTCTTCGTACGAGTACAATTGTGTCTTCACGCAGTGGGGTTCACCATCGTCGCGCTCAACTAGCTCGTCGTCAGAGTCGAGCGGCTTG  1176
      S  V  C  S  K  L  S  D  G  S  S  S  S  Y  E  Y  N  C  V  F  T  Q  W  G  S  P  S  S  R  S  T  S  S  S  S  E  S  S  G  L
1177  AGCGACTTTGAAAGTCCGAGAAGGCCGTGCAATATCTTTGATTACATGCGGCCTCGTGCAATGGATGGTAAGGCCGTTGCTGCTATGCCAAGGCTGACGATTTCGGCACCACCGCATCCA  1296
      S  D  F  E  S  P  R  R  P  C  N  I  F  D  Y  M  R  P  R  A  M  D  G  K  A  V  A  A  M  P  R  L  T  I  S  A  P  P  H  P
1297  CACTGTGCCACTGCTTCGGGTCAAAAGTCCCCTTTCCTGTATGAACTTCAGGACAGTACTTTCTTCGATGGCACACGACTCGACCTCAGCGGCTTGCAGCTCAACCTGGGAGGCTTTGCT  1416
      H  C  A  T  A  S  G  Q  K  S  P  F  L  Y  E  L  Q  D  S  T  F  F  D  G  T  R  L  D  L  S  G  L  Q  L  N  L  G  G  F  A
                                                                                 BamHI
1417  GATGACAAGGACTTTCGAGAAAGTGTTCAGATCGCGCTTAGCATGTCGAGCTCTGAGCAAGGATCCAGCAGAAGCGCGTCATCGAGCTCGTGGGACTCCACGCAGGCGACGACGGATGAC  1536
      D  D  K  D  F  R  E  S  V  Q  I  A  L  S  M  S  S  S  E  Q  G  S  S  R  S  A  S  S  S  S  W  D  S  T  Q  A  T  T  D  D
1537  GATGGCTGGGTCGACGAAGAAGATTTTGATACAGGGTATGCAGCAGCTTGTGATACCAAGCCCATCGACAGAACTCTGTCGGGCCAAGCATCATTCACGCCTCCGGACCACTGGAACAGT  1656
      D  G  W  V  D  E  E  D  F  D  T  G  Y  A  A  A  C  D  T  K  P  I  D  R  T  L  S  G  Q  A  S  F  T  P  P  D  H  W  N  S
1657  AACACTGCGCCCGGTCAAGCATCACGCCAGGAAATCTTTCAAGCTCCCTGTGTTTCTGGATCTCACCCAGCCAATCACAGCCAGACAAGTGCTGACGAGAACCCAAGCATCAGCATCCCG  1776
      N  T  A  P  G  Q  A  S  R  Q  E  I  F  Q  A  P  C  V  S  G  S  H  P  A  N  H  S  Q  T  S  A  D  E  N  P  S  I  S  I  P
                                    <          2
1777  TTCTCTCAGTCTGCACTCTTCGATAGCGATTCATTCGGCCTCGATCAACTCTTCGAGTCTGCCTCAATTCCTGCGCACTTGCCATCGACGCTGTCCCACTCGCAACAGCAAGGCTTTGGA  1896
      F  S  Q  S  A  L  F  D  S  D  S  F  G  L  D  Q  L  F  E  S  A  S  I  P  A  H  L  P  S  T  L  S  H  S  Q  Q  Q  G  F  G
                                 HindIII
1897  CAGATGCCTTTTGTCGACCCAAACATGCAAAGCTTTGAGGAGATCGAACAGGACTTGGACCTCGACATGACCGACATCCAAGAGTTCCTTGGCGGCGAAATTTTTGCTTCCTCCCTCCCC  2016
      Q  M  P  F  V  D  P  N  M  Q  S  F  E  E  I  E  Q  D  L  D  L  D  M  T  D  I  Q  E  F  L  G  G  E  I  F  A  S  S  L  P
                                   PvuII
2017  GGCTCTCAGCAATCGAACGGTTGTGTAGGCTCAGCTGATGCAAATGTACAGGGCAGCAGCGGTGGAGCATGCATGGGAGAAGGTATCCTTCATATGGATTTTGACCCCTACTCGAACTCT  2136
      G  S  Q  Q  S  N  G  C  V  G  S  A  D  A  N  V  Q  G  S  S  G  G  A  C  M  G  E  G  I  L  H  M  D  F  D  P  Y  S  N  S
                                            <          4
2137  TTCTCGCTTGCCTGATGGCGTCGGCTGACTTTGAAAACGCCCACTGGATGGTGCTTTCTAGCGAAAACGTATACCTGCTTCTTCTCTACACTTCTATACCTGGTGGACCATCGACATCGG  2256
      F  S  L  A  *
2257  ATTCGTGTCTCTTTCCTGCTTGCTCCGTTCCACCTCCCTCCGTTGTGCTTTCTTTGGTTCGTTCAAATGTGCTCCTCAATGTGCTTCGTTCAGCTTGTCTACTTTTCACAATTCTCAAAT  2376
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Figure 2. Deletion Mapping of *bW2* and Sequence Analysis

(A) The upper part represents a restriction map of the *bW2* gene (open bar). Only sites used for subcloning are indicated (S, StuI; Ps, PstI; P, PvuII; H, HindIII; B, BamHI; Bg, BglII; E, EcoRI). The stippled bar on the right indicates the N-terminus of the *bE2* gene. Plasmids that carry the fragments indicated are listed on the left. Activity indicates that the respective plasmid caused a Fuz$^+$ Tum$^-$ phenotype when introduced into FBD12-3 (*a1/a2 b1/b1*).

(B) Nucleotide sequence of the *bW2* gene. Note that the orientation of the sequence has been reversed with respect to the orientation of the *bW* gene in all other figures. The translational starts for *bW2* and *bE2* are underlined, as are the two additional in-frame ATG codons of *bW2*. Arrows indicate the direction of transcription. Primers used for PCR amplification of cDNA are indicated as arrows with the respective primer number. Primers 5 and 6 have been used for the amplification of cDNA from *bW1*. Intron–exon borders are underlined.

proposed (Schulz et al., 1990). Why should a strain that is deleted for the ORF *b2* still mate with *b2* specificity? And why should a strain that was *b1* to start with convert to *b2* specificity after replacing the ORF *b1* with the *b2*-del20 sequences? Furthermore, the change in mating specificity of the *b1* strain was observed only when the *b2*-del20 allele had replaced *b1*; strains in which ectopic integration had occurred retained *b1* mating specificity (data not shown). These results made sense only if one postulates the presence of a second gene conferring mating specificity. Since homologous integration was necessary to observe the change in *b* specificity, one had further to assume that the putative gene is not completely contained on the BamHI fragment used for gene replacement (see Figure 1) but extends beyond its boundaries.

To discriminate between 5′ and 3′ location, we constructed *b1/2*-del20 (Figure 1, "b1/2-del 20"), in which the 5′ sequences were derived from the *b1* allele, while sequences downstream of the *b* deletion originated from *b2*. Gene replacements and tests were carried out as described for the derivative *b2*-del20. When *b1/2*-del20 had replaced the *b1* or the *b2* gene, the resulting strains (RK1724 and RK1725) exhibited *b1* mating specificity (Figure 1). This result showed that the presumed gene must be located 5′ of the *b* coding region, since only this region had originated from the *b1* locus in the *b1/2*-del20 construct.

To map this gene more precisely, we generated the *b1*-del30 deletion construct (Figure 1, "b1-del 30"), in which parts of the *b1* gene including 400 bp of 5′ sequences are removed. *b1*-del30 was used for gene replacements in *b1* and *b2* strains as described above. The resulting strains (RK1447 and RK1726) both were completely mating deficient. This suggested that this deletion had affected the activity of both genes, implying that the second gene is located close to the *b* gene. All combinations of strains analyzed for their mating reactions were also tested for pathogenicity in planta. There was a perfect correlation between ability to mate, i.e., to produce the fuzz reaction on plates, and the ability to induce tumors when combined with respective mating partners (Figure 3). None of the strains constructed was able to produce tumors when injected into plants as pure culture.

Cloning and Functional Assay of the Second Gene Encoded by the *b* Locus

Ectopic integrations of the constructs *b2*-del20 or *b1/2*-del20 (see Figure 1) did not confer mating-type specificity, suggesting that the second component of the *b* locus might extend beyond the BamHI site located approximately 1700 bp upstream of the *b* ORF. This region, including 210 bp of the *b2* gene, was cloned to yield plasmid pbW2-1 (Figure 2A) as described in the Experimental Procedures. When pbW2-1 was introduced into a strain heterozygous for *a* and homozygous for *b1* (FBD12-3), transformants exhibited a Fuz^{+} phenotype and were able to induce tumors when injected singly into plants. Transformants in a diploid strain heterozygous for *a* and homozygous for *b2* (FBD11-21), on the other hand, remained Fuz^{-} and were Tum^{-} (data not shown). pbW2-1 thus induces the same phenotypic changes as plasmids carrying the *b2* gene do (Schulz et al., 1990). Since this gene can replace the *b2* gene in transformation experiments, we have decided to designate the new gene *bW2* (the *W* stands for "west") to distinguish it from the *b2* gene cloned first, which we now rename *bE2* (*E* for "east"). Numbers refer to the original numbers of the respective *b* allele.

The nucleotide sequence of the region between ORF *bE2* and beyond the BamHI site, which should contain the *bW2* gene, was determined (Figure 2B). Within the nucleotide sequence two extended regions of potential ORF were found. The first one showed three in-frame ATG codons, while the second one lacked an ATG start codon and extended beyond the BamHI site (Figure 2B). To test whether the discontinuity could indicate the presence of an intron, two sets of oligonucleotides covering the putative coding region (see Figure 2B) were used in the polymerase chain reaction (PCR), employing cDNA as template (see the Experimental Procedures for details). The products were cloned to yield plasmids pbW2-c1 and pbW2-c2 (Figure 2A) and sequenced. This allowed us to precisely map a 270 bp intron (Figure 2B) and showed, furthermore, that a transcript encompassing the ORF is present. This transcript must be of low abundance, as we were unable to find a *bW2* cDNA clone in a λgt10 library. Since plasmids pbW2-3 and pbW2-4, which carry *bW2* fragments lacking the first and second possible ATG start codon, respectively, were both active in transformation (Figure 2A), translation of *bW2* that is initiated at the third in-frame ATG (position 1 in Figure 2B) must produce a functional product (although, from the sequence context, the ATG at position −58 shows the best fit to the consensus sequence preceding translation initiation codons in filamentous fungi [Ballance, 1991]). The predicted polypeptide of *bW2* therefore consists of 626 amino acids with a molecular weight of 69 kd. The C-terminal 33 amino acids can be deleted without noticeable effects on activity of the product (Figure 2A, plasmid pbW2-2), while no activity was observed for a fragment truncated at both the C- and N-terminal ends of the *bW2* coding region (Figure 2A, plasmid pbW2–5). A characteristic feature of the *bW2* sequence is a motif related to the homeodomain (Figures 4 and 5).

Comparison of Four *bW* Alleles

The *bW* gene must exist in at least two alleles, since the gene replacement experiments had indicated that the respective region confers *b2* or *b1* specificity when derived from *b2* or *b1* strains, respectively. We have, therefore, analyzed three additional *bW* genes. The entire coding region for *bW1* was sequenced, and a partial cDNA clone was generated (pbW1-c1, see the Experimental Procedures for details). The nucleotide sequence of the *bW1* cDNA revealed the presence of an intron in *bW1* at the same position as in *bW2* (not shown). For the analysis of *bW3* and *bW4*, we have restricted our sequence analysis to the N-terminal portion of the respective genes up to the BamHI site, for the following reason. The gene replacement experiments with the deletion derivatives *b2*-del20 and *b1/2*-del20 had indicated that allele specificity in the *bW* genes is determined by sequences between the

Figure 4. Amino Acid Comparison of Four *bW* Genes

Amino acids sequences of *bW1*, *bW2*, *bW3*, and *bW4* are aligned. Sequence identity with *bW1* is indicated by a dash. Sequences for *bW3* and *bW4* were determined only up to the BamHI site (see Figure 2). Asterisks mark the motif related to the homeodomain.

BamHI site and the translational start of *bW*. The amino acid alignments revealed, indeed, that allelic differences are very prominent in the N-terminal 130 amino acids (44% conserved), while the C-terminal portions showed 96% conservation (Figure 4). Up to the BamHI site all four amino acid sequences can be aligned without gaps; the N-termini differ in length by 1 amino acid (Figure 4). The overall organization of *bW* polypeptides is thus highly reminiscent of *bE* polypeptides, in which different alleles show 63% identity in the N-terminal 110 amino acids and are conserved to 93% for the C-terminal domain (Schulz et al., 1990; Kronstad and Leong, 1990). Despite this similarity, the predicted polypeptide sequences for *bW* and *bE* have very little in common, however. The only short stretch of similarity comprises the homeodomain-related motif (Figure 5). In *bW* this motif spans the intron–exon borders. The

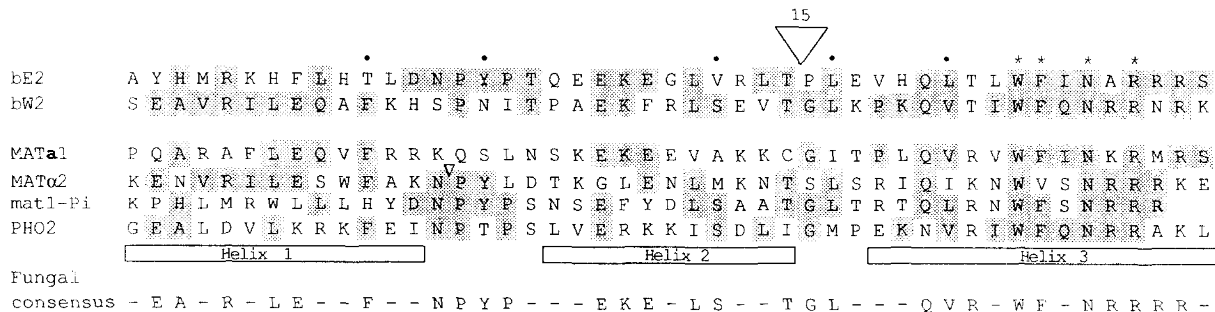

Figure 5. Comparison of Homeodomain-Like Motifs in *bW2* and *bE2* with Homeoboxes from Yeast Regulatory Proteins

The amino acid sequence in the homeodomain of *bW2* (from position 145 to 191) and *bE2* (from position 119 to 180) (Schulz et al., 1990) is compared with those contained in the yeast family consisting of MATa1, MATα2, mat1-Pi, and PHO2 (see Scott et al., 1989 for references). Amino acid identities between *bW2* and *bE2* with any of the four yeast sequences is stippled. A 15 amino acid stretch has been looped out from *bE2* to maximize sequence identities. The small triangle in the MATα2 sequence marks the position where three amino acids (IEN) have been looped out to maximize identities. The fungal consensus indicates three or more sequence identities at a given position for all six sequences shown. The boundaries of the three α helices 1, 2, and 3 that are involved in DNA binding in MATα2 (Wolberger et al., 1991) are indicated. Asterisks mark the four amino acids that are invariant in all nonyeast homeodomains, and dots denote amino acid positions that are highly conserved (see Scott et al., 1989); of these only the conserved position in helix 2 is different in the fungal consensus.

bW and *bE* genes are arranged divergently. Transcription of both genes must initiate from an intergenic region of only 260 bp which, furthermore, is part of the variable region with 72% nucleotide conservation (not shown). In the untranslated regions of the *bW* genes, CAAT and TATA boxes are absent, as has been observed for many genes in filamentous fungi (see review by Ballance, 1991). There are, however, pyrimidine-rich regions between positions −33 and −40, positions −61 and −89, and positions −108 to −120 in *b2W* (Figure 2B), which are highly conserved in the other alleles and could conceivably contribute to precise transcription initiation (Hamer and Timberlake, 1987).

Disruption of *bW* Genes Does Not Affect Mating Specificity

To determine more precisely the contributions of *bW* and *bE* genes to pathogenic development, we have disrupted the *bW1* gene by insertion of a hygromycin cassette, leaving *bE1* intact (Figure 1, "b1-del 40"). The *b1*-del40 construct was introduced in FB2 (*a2 b2*) and FB6b (*a1 b2*). Strains in which gene replacement had occurred displayed the mating specificity of *b1* (Figure 1). To obtain a strain lacking *bW2*, we generated a deletion that removes the ORF except for the C-terminal 80 amino acids (Figure 1, "b2-del 50"). The *b2*-del50 construct was introduced into *b1* strains (FB6a and FB1). Transformants in which gene replacement had taken place exhibited the mating specificity of *b2* (Figure 1). These results demonstrate that strains deleted for *bW* display a mating specificity that is determined by the allele of the *bE* gene that is introduced with the deletion construct. Together with the experiments presented above, these results show conclusively that mating specificity of a given *b* locus can be conferred by either the *bW* or the *bE* gene.

One Pair of *bW* and *bE* Genes Originating from Different Alleles of the *b* Locus Suffices for Development

The experiments performed so far left several possibilities for how *bE* and *bW* need to be combined (see Figure 3A, parts 2 to 4): two different *bW* as well as two different *bE* or a *bW–bE* combination could trigger development. With the availability of deletion derivatives in which only the *bW1*, *bW2*, *bE1*, or *bE2* gene is intact, we could now ask whether one can initiate pathogenic development by combining haploid lines that each contribute only one component of the *b* locus. Strains with compatible *a* alleles were crossed and assayed for the fuzz reaction on plates as well as for tumor development in planta. Matings between strains carrying different *bW* alleles were unsuccessful (Figure 3A, part 6 and Figure 3B) as were all combinations of strains carrying different *bE* alleles (Figure 3A, part 7 and Figure 3B). Combinations of *bW1* with *bE1* and combinations of *bW2* with *bE2* were defective in initiating development, as expected from the behavior of haploid lines (Figure 3B). However, by combining *bW1* with *bE2* or *bW2* with *bE1* (Figure 3A, parts 8 and 9 and Figure 3B) the developmental program was initiated. This proves that *bE* and *bW* genes function as pairs and trigger development only when derived from different alleles of the *b* locus.

Phenotype of a Haploid Strain with a Combinatorial *b* Locus

Having established that fusion of two haploid strains each providing only one component of the *b* locus can result in pathogenic development, we next determined whether a haploid strain in which one of the genes in the *b* locus is replaced by an allele from a different *b* locus is pathogenic. To this end a combinatorial *b* locus encoding *bW1* and *bE2* was constructed on plasmid pbW1/E2 and introduced by homologous integration into *b2* strains differing in their *a* alleles (see the Experimental Procedures for details). The resulting strains (RK1659 and RK1645) were able to mate with *b1* as well as with *b2* strains (Figure 6B). When they were spotted on charcoal nutrient media alone (Figure 6B), colonies developed a weak Fuz^+ phenotype, which was more prominent in the *a1* than in the *a2* genetic background. Both strains could be crossed, although they were identical at *b* (Figure 6B). A full Fuz^+ phenotype was also observed when they were crossed with strains that lacked both *bE* and *bW* and contributed only *a1* or *a2*, respectively (Figure 6B). These experiments suggested that the combinatorial locus containing *bW1* and *bE2* provides an active regulatory species in the haploid. At the same time these experiments reinforced the contribution of the *a* locus to filamentous growth (Banuett and Herskowitz, 1989). When assayed in planta, strains containing the combinatorial *b* locus induced tumors when injected as pure cultures (Figure 6A). In size and time of appearance these tumors were indistinguishable from tumors induced by solopathogenic diploid strains homozygous for *a* and heterozygous for *b* (FBD11-7 and FBD12-17). These results demonstrate that combinatorial interactions of just one *bW* and another *bE* allele in a haploid cell are sufficient to turn on pathogenic development.

Discussion

Studies described in this paper have revealed that the *b* locus of U. maydis contains a second gene, which we term *bW*; the previously cloned *b* genes are now designated *bE*. The organization of the whole *b* locus is depicted schematically in Figure 7A. There appear to be as many alleles of *bW* as there are of *bE*. Only pairwise combinations of *bE* and *bW* genes derived from different alleles of the *b* locus trigger pathogenic development. In regular crosses between haploid strains in which each mating partner contributes one pair of genes, two new pairs of polypeptide products are generated, and both are active. The redundance in having two pairs with the same function can be viewed as a safeguarding device for the switch to sexual development. Since the products of *bE* and *bW* are both necessary to initiate development, it is likely that they function as gene activators, although the formal possibility that they act as repressor of a repressor for pathogenic development (Banuett, 1991) cannot be excluded.

The presence of homeodomain-related sequence motifs

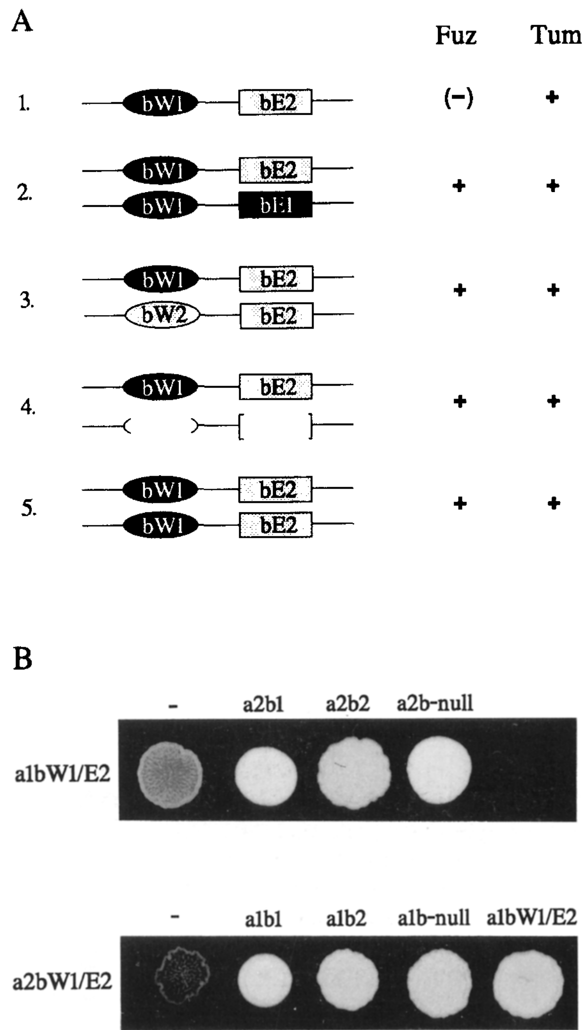

Figure 6. Mating Specificity of Haploid Lines Containing *bW1*/E2

(A) Parts 1 to 5 show schematic representations of allele combinations used in crosses; parentheses indicate that the respective allele was eliminated by gene disruption. *a1* and *a2* strains with the indicated stucture of the *b* locus were tested. All crosses were performed with strains of opposite *a* mating type. The Fuz phenotype was determined on charcoal nutrient plates; the Tum phenotype was assessed by injecting strains into maize plants. Fuz (−) indicates that short filaments were visible.

(B) Plate mating reactions of strains carrying the *bW1*/E2 locus. Strains indicated on the left were spotted alone or in the combination shown above on charcoal plates. A more detailed description of the strains used can be found in Table 1.

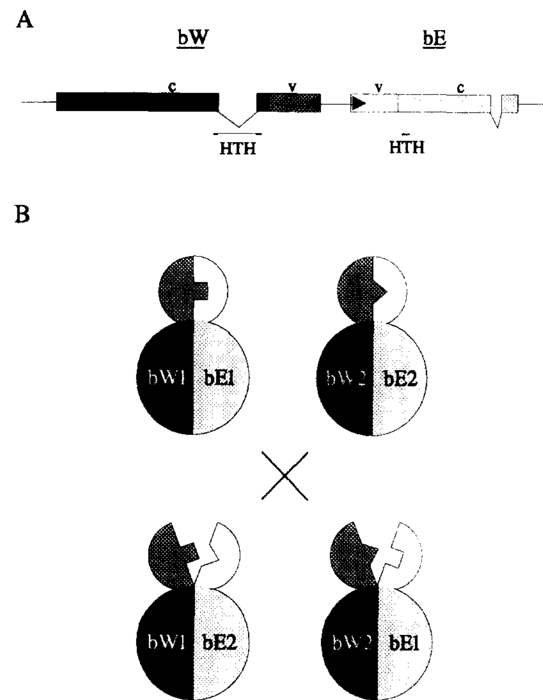

Figure 7. Organization of the *b* Locus and Model for the Interaction of *bW* and *bE* Polypeptides

(A) *bW* and *bE* genes are drawn to scale. Variable (v) and constant (c) regions are indicated by different shading. Intron sequences are indicated. Arrows denote the direction of transcription. HTH marks the motif related to the homeodomain. Note that the HTH motif in *bW* is disrupted by the intron.

(B) *bW* and *bE* polypeptides are proposed to interact. In the haploid cell the interaction of *bW* and *bE* polypeptides from the same *b* allele produces heterodimers that have evolved to be inactive, presumably because the DNA-binding domains are buried by interactions between the variable domains of both polypeptides (top left and right). Upon mating, two additional new types of heterodimers are formed (bottom left and right) in which the DNA-binding motifs are exposed and which are both competent to activate the same set of genes.

in *bE* (Schulz et al., 1990) and *bW* suggests that both polypeptides act as transcriptional regulators. The motifs are located at similar distances from the N-terminus of both polypeptides (Figure 7A). The best alignment of *bE* and *bW* homeodomain-related sequences requires looping out a stretch of 15 amino acids from *bE* as has been done, to maximize similarities between *bE* and the yeast and fission yeast homeodomain-related regulatory proteins encoded by *MAT*a1, *MAT*α2, *mat*1-Pi, and *PHO2* (Schulz et al., 1990; see Figure 5). The loop is in a position between putative helices 2 and 3 (Figure 5) and shows amino acid variation between different *bE* alleles (Schulz et al., 1990). In the same position, a 21 amino acid segment has also been looped out to maximize similarity between the homeodomain of the transcription factor LF-B1 and a representative group of other homeodomains (Finney, 1990). The homeodomain-related motifs in the four *bW* alleles analyzed are identical. The homeodomains of *bE* and *bW* differ in two positions that may be crucial for their function. One is the third position of the WF-N-R motif (the amino acid between F and N; see Figure 5), which Treisman et al. (1989) have demonstrated determines DNA binding specificity. The other is an NPYP motif, which is characteristic for three of the four yeast homeodomains (Figure 5). It is located between helix 1 and helix 2. Helix 1 does not contact DNA but stabilizes the helix-turn-helix conformation of helices 2 and 3 (Kissinger et al., 1990; Qian et al., 1989; Wolberger et al., 1991). Interestingly, the MATa1 and MATα2 homeodomains also display differences at

these locations (see Figure 5). MATa1 has been shown to alter the DNA binding specificity of the MATα2 polypeptide by forming a heterodimeric complex (Goutte and Johnson, 1988; Dranginis, 1990). It is hence tempting to speculate that an association of *bE* and *bW* polypeptides in U. maydis might also result in a complex in which both partners contribute to binding site selection — as is the case for several other heterodimers, like MyoD-E2A and Myc-Max, which direct development (Blackwell and Weintraub, 1990; Blackwood and Eisenman, 1991).

We propose that the *bE* and *bW* polypeptides form heterodimers (see Figure 7B). If self/nonself discrimination occurs at this level, then heterodimers between *bE* and *bW* polypeptides from the same *b* allele must be inactive, while *bE–bW* heterodimers derived from different alleles of the *b* locus trigger development. If *bW* and *bE* polypeptides from the same *b* locus associate, it is feasible that their variable domains adopt a specific conformation that inhibits the ability of the complex to bind to DNA (Figure 7B). We favor the idea that it is the DNA-binding properties in which the active and the inactive complex differ, because otherwise the binding sites in the dikaryon should still be occupied by the inactive heterodimer and compete with the active form. The dimerization domains in *bE* and *bW* are then likely to be located in the constant regions of both polypeptides, and the variable domains of the *bW* and *bE* polypeptides become prime candidates for additional contacts between *bE* and *bW*. The nature of such additional contacts could determine whether the DNA-binding domains are exposed or buried (Figure 7B), i.e., whether the complex is active or inactive.

We then need to explain why the complex that forms in haploid strains is inactive. We suggest that it may be evolutionarily disadvantageous to form an active heterodimer in haploids, because such cells would have lost the requirement to mate. In addition we have observed that haploids with a pair of *bE* and *bW* genes, which form an active complex, produce smaller galls and fewer spores on certain lines of corn (R. K., unpublished data). Therefore, although the exact nature of the driving force is unclear, we suggest that the inactive complex is the evolutionary result of selection against the active form. The variability of the N-terminal domains therefore reflects the variety of different solutions for a rather tricky problem: to create by specific interactions an inactive complex of two proteins and at the same time to guarantee that in combinations with other *bE* or *bW* polypeptides an active complex is formed. Having two multiallelic genes rather than one as assumed in our previous models (Schulz et al., 1990) reduces the complexity of this task, because both polypeptides can accommodate different structural changes without affecting function. By this model the existence of so many different *b* alleles can be explained (at least 33 different *b* mating types have been isolated from nature [DeVay, quoted in Wong and Wells, 1985]). The chance that an inactive complex is formed by the association of *bE* and *bW* polypeptides from different alleles is probably small, and if it occurs at all then the existence of the second active pair could guarantee sexual development. The assertion that rather specific interactions are required for silencing (see Figure 7B) is supported by an observation that even subtle changes in one of the components converts the complex to the active form. We have recently shown that a mutant derivative of *bE3*, with just one amino acid change in the variable domain, is active in turning on the developmental program in combination with *bW3* (F. Schauwecker and R. K., unpublished data).

During meiosis, rare recombination events between the variable regions could create a hybrid *b* locus whose products turn on development, as in the haploid *bW1*/E2 strains created artificially. The same effect could result from point mutations in the variable domains of either *bW* or *bE*. If there is selective pressure against the active *bW–bE* complex, the proposed mutations and compensatory mutations could eventually create a *b* allele with a new specificity. The high probability of going through a stage in which the heterodimer is active in the haploid strain may be one of the reasons why direct attempts to find strains with a new *b* specificity have been unsuccessful (Puhalla, 1970). A mutant isolated by Banuett (1991), which bypasses the requirement for a different *b* locus in matings and thus behaves like the haploid strains with a combinatorial *b* locus, is closely linked to *bE1* and could, therefore, conceivably map in *bW1*. This mutation is recessive, however, which we would not predict if the mutation had changed the specificity of *bW1*. It is therefore more likely that the mutation has affected a gene that is controlled by the *b* locus (Banuett, 1991).

There appear to be intriguing mechanistic parallels to other basidiomycete fungi like Coprinus cinereus and Schizophyllum commune, which both have a multiallelic mating type system at two unlinked loci. Recent molecular studies indicate that at least two nonallelic mating type genes must cooperate to activate Aα development in Schizophyllum commune (Ullrich et al., 1991). For the *A* locus in Coprinus cinereus, which contains two subunits with several genes, the introduction of only one nonallelic gene for one of the subunits is sufficient to trigger *A*-regulated development (Mutasa et al., 1990; May et al., 1991). It is perhaps not surprising that these genes encode polypeptides that are very different in their primary sequence but all contain homeodomain-related DNA-binding motifs (L. Casselton, R. Ullrich, and C. Novotny, personal communication). It will be an exciting task for further experimentation to define in biochemical terms which structural elements govern association and activity of the *bE* and *bW* polypeptides. Such studies should also provide a powerful tool to identify the target(s) for the active heterodimeric *bE–bW* complex.

Experimental Procedures

Strains

Cloning in Escherichia coli was done in DH5α (Φ80 *lacZΔM15*) Δ[*lacZYA-argF*] U196 *recA1 endA1 hsdR17* r^-m^+ *supE44 thi1 gyrA relA1* and the DH5α-derived strain DH5α*mcr* (both obtained from Bethesda Research Laboratories). U. maydis strains used and constructed are listed in Table 1.

Growth Conditions for U. maydis

CM and YEPS were prepared as previously described (Holliday, 1974; Tsukuda et al., 1988). Strains were grown at 28°C or 32°C. Yeast-like

Table 1. U. maydis Strain List

Strain	Relevant Genotype	Reference
FB1	*a1 b1*	Banuett and Herskowitz, 1989
FB2	*a2 b2*	Banuett and Herskowitz, 1989
FB6b	*a1 b2*	Banuett and Herskowitz, 1989
FB6a	*a2 b1*	Banuett and Herskowitz, 1989
FBD11	*a1 a2/b1 b2*	Banuett and Herskowitz, 1989
FBD11-7	*a1 a1/b1 b2*	Banuett and Herskowitz, 1989
FBD12-17	*a2 a2/b1 b2*	Banuett and Herskowitz, 1989
FBD11-21	*a1 a2/b2 b2*	Banuett and Herskowitz, 1989
FBD12-3	*a1 a2/b1 b1*	Banuett and Herskowitz, 1989
RK1722	*a2 bW2*	*b2*-del20 allele in FB2, this work
RK1723	*a1 bW2*	*b2*-del20 allele in FB1, this work
RK1724	*a2 bW1*	*b1/2*-del20 allele in FB2, this work
RK1725	*a1 bW1*	*b1/2*-del20 allele in FB1, this work
RK1607	*a1 bE1*	*b1*-del40 allele in FB6b, this work
RK1610	*a2 bE1*	*b1*-del40 allele in FB2, this work
RK1661	*a2 bE2*	*b2*-del50 allele in FB6a, this work
RK1662	*a1 bE2*	*b2*-del50 allele in FB1, this work
RK1447	*a1 b*-null	*b1*-del30 allele in FB1, this work
RK1726	*a2 b*-null	*b1*-del30 allele in FB2, this work
RK1659	*a2 bW1/E2*	*bW1*/E2 allele in FB2, this work
RK1645	*a1 bW1/E2*	*bW1*/E2 allele in FB6b, this work

or mycelial phenotypes were distinguished on charcoal nutrient medium (Holliday, 1974; Banuett and Herskowitz, 1989). For mating strains were cospotted on charcoal nutrient plates and incubated at room temperature for 1–4 days.

Pathogenicity Test

To assay pathogenicity, 8- to 10-day-old corn seedlings (Aztec and/or Early Gold Bantam), grown in a greenhouse under controlled conditions (14 hr light, 28°C; 10 hr dark, 20°C), were infected with pure cultures as described by Puhalla (1968). For strains that were pathogenic, of six plants infected, four to six plants developed tumors after 5–8 days (Tum$^+$ phenotype); in nonpathogenic lines or incompatible interactions, none of the six plants infected developed tumors (Tum$^-$ phenotype).

DNA Procedures

Isolation of U. maydis DNA was done as described previously (Schulz et al., 1990). Standard procedures for Southern blotting on Hybond N$^+$ nylon filters (Amersham) were employed (Sambrook et al., 1989). Radioactive labeling of DNA fragments was performed by primer extension as previously described (Feinberg and Vogelstein, 1983). Nucleotide sequences were determined by the dideoxynucleotide chain termination method of Sanger et al. (1977). Alkali-denatured plasmid DNA was sequenced using T7 DNA polymerase (T7 Sequencing Kit, Pharmacia). DNA was either subcloned in pTZ18R or sequenced by designing specific primers. The *bW2* gene was sequenced from plasmids pUb2 and pbW2-1. The *bW1* gene nucleotide sequence was derived from plasmids pUb1 and pbW1-SS. The partial nucleotide sequences for *bW3* and *bW4* were derived from pUb3 and pUb4, respectively. All nucleotide sequences were determined for both strands.

Transformation of U. maydis

Transforming DNA was linearized with appropriate restriction enzymes. Transformation of U. maydis was performed as described by Schulz et al. (1990). To regenerate protoplasts, transformation mixtures were plated directly on plates containing 10 ml of 1.5% agar in YEPS, 1 M sorbitol, 400 μg/ml hygromycin overlaid with 10 ml of 1.5% agar in YEPS, 1 M sorbitol, shortly before use.

Plasmids and Plasmid Constructions

pTZ18R, pT7T318U, pSL1180 (Pharmacia), and pSP72 (Promega) were used for cloning DNA fragments. pHLN is a pUC12 derivative carrying the hygromycin B resistance gene under control of U. maydis *hsp70* regulatory sequences (Wang et al., 1988) with a NotI site in the polylinker (Schulz et al., 1990). pHLNH is identical to pHLN, except that the HindIII site downstream of the hygB gene was destroyed by filling in the protruding ends. The hygB cassette including the U. maydis *hsp70* promoter can be excised from pHLN with HindIII or PvuII.

pUb1, pUb2-2, and pUb3 carry 8 kb BamHI fragments, and pUb4 carries a 4.6 kb BamHI fragment of alleles *b1*, *b2*, *b3*, and *b4*, respectively, cloned in pHLN (Schulz et al., 1990). On these fragments the ORF *bE* is intact, while the ORF *bW* terminates at the BamHI site. pFB1 carries the 8 kb BamHI fragment of pUb1 cloned into the BamHI site of pUC18 and was kindly provided by F. Banuett.

The pbW2 plasmid series described below is depicted schematically in Figure 2A.

pbW2-1: a 5.5 kb StuI fragment carrying *bW2* was isolated from a *b2* cosmid clone p16C5-7 kindly provided by F. Banuett. This fragment, which extends from a StuI site in *bE2* (position 210) beyond the BamHI site situated in the *bW2* gene, was inserted into the HincII site of pT7T318U. Subsequently the hygB cassette was excised as PvuII fragment from pHLN and cloned into the SmaI site of the polylinker.

pbW2-2: A 2.6 kb StuI–PvuII fragment was excised from pb2W-1 as XbaI–PvuII fragment and cloned into the respective sites of pSP72. In a second step the hygB cassette was excised as PvuII fragment from pHLN and cloned into the PvuII site.

pbW2-3: A 3.2 kb EcoRI–PstI fragment of pbW2-1 was ligated into the respective sites of the pSP72 polylinker. Subsequently a PvuII fragment containing the hygB cassette was inserted in the EcoRV site of the polylinker.

pbW2-4: pbW2-3 was cleaved with BglII, and a fragment extending from the BglII site in the polylinker to a BglII site in *bW2* was isolated and ligated to a 936 bp fragment containing the 5′ end of the *bW2* gene in the orientation that restores the ORF *bW2*.

pbW2-5: The 1.7 kb HindIII fragment of pbW2-1 was cloned into the HindIII site of pHLNH.

pbW1-SS: StuI fragments in the size range 3.5 to 4.5 kb were isolated from a genomic digest of FB1 DNA and cloned into the StuI site of pSL1180. A clone hybridizing to a C-terminal fragment of *bW2* was isolated. The cloned 4 kb StuI fragment extends from a StuI site in *bW1* to a StuI site downstream of *bW1*.

Constructs for Gene Replacements

To generate the constructs for homologous recombination, it was necessary to subclone fragments first into various polylinker regions to be able to excise them with protruding ends facilitating the construction. Here we omit most of the subcloning steps and describe only the final constructs, which are outlined schematically in Figure 1.

pb2-del20: A 1.7 kb BamHI–HindIII fragment comprising the first 10 amino acids of the ORF *bE2* and upstream sequences was cloned as BamHI–EcoRV fragment in pSP72. At the EcoRV site this fragment was connected to a 4 kb EcoRV–BamHI fragment comprising sequences from an ApaLI site downstream of the ORF *bW2* to the next BamHI site. Into the EcoRV site of this plasmid a PvuII fragment carrying the hygB resistance was inserted.

pb1/2-del20: This construct is similar to pb2-del20 except that the 1.7 kb BamHI–HindIII fragment originated from plasmid pUb1 carrying the *b1* allele.

pb1-del30: pFB1 was cleaved with BglII and subjected to a partial digest with XbaI to remove a fragment encompassing 678 bp of the *bE1* ORF and 410 bp of the 5′ flanking sequence. This fragment was replaced with a hygB PvuII cassette isolated as a BamHI–XbaI fragment.

pb1-del40: The PvuII hygB cassette of pHLN was ligated to BamHI linkers, cleaved with BamHI, and inserted into the unique BglII site located in the *bW1* gene of pFB1.

pb2-del50: A 2.3 kb SalI fragment encompassing the 3′ end of *bW2* and downstream sequences was cloned into the respective sites of pSP72. Next, a 2.4 kb EcoRI fragment carrying ORF *bE2* was isolated from pUb2-2 and inserted into the EcoRI site of the previous plasmid followed by inserting the PvuII hygB cassette into the SmaI site.

pbW1/E2: A 5 kb BamHI–XbaI fragment comprising a truncated *bW1* gene including 288 bp upstream of the translational start and the hygB cassette was excised from pb1/2-del20 and cloned into the respective sites of pSP72. In the second step a 2.4 kb EcoRI fragment with the ORF *bE2* and upstream sequences of 125 bp was isolated from pUb2-2 and cloned into the respective site of pSP72; the fragment

was subsequently excised as SmaI–EcoRV fragment and cloned into the PvuII site of the plasmid carrying the N-terminal portion of *bW1* and the hygB cassette. The orientation of fragments relative to each other is such that *bW1* (truncated) and *bE2* are arranged divergently with the hygromycin cassette in between. When this construct replaces *b2*, one of the crossover sites must lie in the constant portion of the *bW* genes between variable domain and BamHI site. Homologous integration therefore generates a full-length *bW1* gene.

Plasmids were cleaved with BamHI prior to transformation in U. maydis, except that pb2-del50 was cleaved with EcoRV.

Verification of Gene Replacement

DNA from transformants was isolated, cleaved with BamHI, and subjected to Southern analysis, using as probe the 8 kb BamHI fragment of pUb2-2 comprising the *b2* locus. *b1* and *b2* strains show a hybridization signal at 8 kb. In strains in which the resident *b* locus was replaced by the constructs shown in Figure 1 or the composite *b* locus *bW1/E2*, the 8 kb fragment was absent, and hybridization signals expected from the size of the deletion and hygB substitution were obtained.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from U. maydis strains FBD11 (*a1/a2 b1/b2*) and FBD11-21 (*a1/a2 b2/b2*) by the glass bead–phenol method developed for yeast according to Sprague et al. (1983). Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography as described (Sambrook et al., 1989). Double-stranded cDNA was synthesized according to the method of Gubler and Hoffmann (1983) using a cDNA synthesis kit from Amersham. cDNA clones of *bW* were generated by PCR amplification of cDNA using the following primers:

Primer 1: 5′-ATGCTACCGCCACTGCCAG-3′
Primer 2: 5′-GATCGAGGCCGAATGAATC-3′
Primer 3: 5′-GAGAAGTTCCGACTTTCAG-3′
Primer 4: 5′-GAAAGCACCATCCAGTGGGCG-3′
Primer 5: 5′-CTAGAGGGTCCGAACCAAC-3′
Primer 6: 5′-GCCATCGCTTAGTTTCGAAC-3′

Priming sites are indicated in Figure 2B. The amplification reaction was done according to Saiki et al. (1988). Template DNA was cDNA from FBD11-21 and FBD11, respectively. Amplification reactions contained approximately 10 ng of cDNA and 50 pmol of each primer, dNTPs, buffer, and Taq polymerase as recommended by Perkin Elmer Cetus usng 50 cycles of 1 min at 93°C, 1 min at 60°C, and 3 min at 72°C in a Coy Tempcycler, followed by a general extension step of 10 min at 72°C. PCR products were gel purified, blunted with T4 polymerase, kinased, and cloned into the HincII site of pTZ18R. From FBD11 cDNA a 0.7 kb fragment originating from *bW1* was amplified using primers 5 and 6 to yield pbW1-c1. From FBD11-21 cDNA *bW2* clones pbW2-c1 and pbW2-c2 were generated, which contain a 1.6 kb fragment (primers 1 and 2) and a 1.4 kb fragment (primers 3 and 4), respectively. All inserts were sequenced and revealed the intron indicated in Figure 2.

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The accession numbers for the sequences reported in this article are M84179 (*bW1*), M84180 (*bW3*), M84181 (*bW4*), and M84182 (*bW2*).